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STUDIES ON THE ACTIVE SITES OF THE CHYMOTRYPSINS

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "STUDIES ON THE ACTIVE SITES OF THE CHYMOTRYPSINS", submitted by Kenneth James Stevenson in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

ABSTRACT

The active sites of CHT-A₄ and CHT-B have been investigated through chemical labelling techniques using bifunctional reagents and through the isolation and characterization of segments of the important active site region in the enzymes.

The family of "serine" esterases and proteases is characterized by their ability to react with diisopropylphosphofluoridate (DFP) at a unique serine residue found in the sequence GLY-ASP-SER-GLY. In addition to serine, a histidine residue has been repeatedly implicated in enzyme catalysis. Recently, four members of this interesting family of enzymes, CHT-A₄, CHT-B, trypsin and elastase, have been demonstrated to possess numerous structural homologies.

The considerable homologies which exist in proximity to the disulfide bridges suggested that the tertiary structures of the enzymes studied to date are very similar. Moreover, the "active" serine residue and a pair of histidine residues have been shown to be closely associated with the disulfide bridges. Since the chymotrypsins, trypsin and elastase differ in their substrate specificity, it appeared that the distinguishing feature amongst these enzymes was the nature of their substrate binding site.

Pertinent to the present studies was the role of the "histidine-loop" in the enzymic activity of the trypsin, CHT-A₄ and CHT-B. This structural feature has been postulated

to be the major (specific)* substrate binding site in trypsin and chymotrypsin A₄. Inconsistencies in the hypothesis and earlier observations in this laboratory on the deacylation rates of the chymotrypsins, prompted the elucidation of the sequence of the "histidine-loop" in CHT-B.

To facilitate the sequence elucidation of this region, a method has been employed which enabled the isolation of the "histidine-loop" as an intact peptide, thereby eliminating the tedious job of isolating fragments of this region from a complex mixture of peptides. The "histidine-loop", formed by the disulfide bridge 42 to 58, was isolated by first reducing CHTG-B with mercaptoethanol followed by alkylation of the sulfhydryl groups with ethylenimine. The newly formed amino acid —S-(β-aminoethyl)-cysteine— possessed a similar structure to lysine and was therefore able to serve as a substrate for trypsin. As a result, tryptic digestion of S-(β-aminoethyl)-CHTG-B led to the recovery of the "histidine-loop" peptide through cleavage of the bonds adjacent to the modified cysteine residues 42 and 58.

Elucidation of the amino acid sequence of the "histidine-loop" of CHTG-B revealed that it was strikingly homologous to the corresponding structure in CHTG-A and trypsinogen. Variation in the amino acid sequence and in the nature of the amino

* Given the general substrate $R-CH_2-\underset{\substack{| \\ NH \\ | \\ R^1}}{CH}-CO-X$, the specific

binding site accommodated R through hydrophobic bonding, whereas R¹-NH- is accommodated in the acylamido binding site through a combination of hydrogen and hydrophobic bonds.

acid was permitted only in a segment comprised of three residues (48-50) located mid-way between the disulfide bridge. The presence of one and two acidic residues in this segment of CHTG-A and CHTG-B, respectively, accounted for the hydrophilic character of the "histidine-loop" of these enzymes. On the other hand, the corresponding structure in trypsin was void of charged residues and thus was predominantly hydrophobic in nature. The hypothesis that the "histidine-loop" was the major substrate binding site was not supported by the present studies since the nature of the "histidine-loop" did not complement the enzyme substrates. It is possible that a portion of the "histidine-loop" could comprise a segment of the subsidiary site known as the acylamido binding site. Candidates for the specific substrate binding site have been proposed where homologous, predominantly hydrophobic, sequences contain an acidic residue in trypsin which is absent in the chymotrypsins. It appears that the "histidine-loop" is required to maintain the proper stereochemical relationship between the two histidine residues in the active site of the "serine" enzymes.

Bifunctional reagents have been used to probe the active site of the chymotrypsins. The reagents utilized in the present study possessed two functional groups: an aromatic moiety capable of being absorbed to the binding site and a reactive chloromethyl ketone moiety capable forming a covalent linkage with a residue in the active site. The inhibition of

CHT-A₄ by the bifunctional reagent L-1-tosylamido-2-phenylethyl chloromethyl ketone (L-TPCK, C₆H₅.CH₂CH(NHSO₂C₇H₇).CO.CH₂Cl) was previously shown to be associated with the loss of histidine-57 present in the "histidine-loop". In addition, the alkylation of CHT-A₄ by 1,2-epoxy-3-phenoxypropane (EPOP, C₆H₅.O.CH₂.CH^O.CH₂) resulted in the stoichiometric S-alkylation of methionine-192. For the purpose of elucidating those parts of the alkylating group responsible for directing the alkylation to a histidine or methionine residue, phenoxymethyl chloromethyl ketone (PMCK, C₆H₅.O.CH₂.CO.CH₂Cl) was synthesized. The D-isomer of TPCK was also investigated for inhibitory capabilities in order to determine the role of the asymmetric carbon.

A homologous series of aromatic chloromethyl ketones (C₆H₅.[CH₂]_n.CO.CH₂Cl, α-chloroacetophenone (CA, n = 0), benzyl chloromethyl ketone (BCK, n = 1), and β-phenylethyl chloromethyl ketone (βPECK, n = 2) has been studied in an attempt to further elucidate the structural requirements for histidine alkylation. The aromatic moiety of the reagents was expected to be bound in a common hydrophobic binding site thus placing the reactive chloromethyl ketone group at various positions in the active site.

A detailed study of the effect of pH on the apparent first-order rate constant (k_{obs}) of inhibition of the chymotrypsins by L-TPCK and PMCK has been presented. The bell-shaped curve obtained for the inhibition of CHT-A₄ with L-TPCK possesses a pH-optimum of 7.7 to 8.0 and was dependent on apparent pK_a's of 6.5 and 9.0. The pH-optimum obtained for CHT-B inhibited with L-TPCK was found to be quite broad and extended from approximately pH 7.2 to 8.0. The curve was dependent on apparent pK_a's of 6.3 and

9.0. The ascending arm of the bell-shaped curves, depicting the effect of pH on the rate of inhibition of CHT-A₄ and CHT-B with L-TPCK, corresponded to a theoretical titration curve, and undoubtedly represented the ionization of the imidazole group of histidine-57. Of particular interest was the observation that the descending arm of the bell-shaped curves (k_{obs} versus pH) was dependent on a group with apparent $pK_a \sim 9$. Studies presented herein refuted the possibility that instability of L-TPCK in alkaline medium or the ionization of the tosylamido amido group accounted for the decrease in inhibition. It is felt that the ionization of the α -amino group of isoleucine-16, the N-terminal of the B-chain, has an unfavourable influence on the binding of L-TPCK to the chymotrypsins which results in the observed decrease in the rate of inhibition. Support for this theory was obtained from a plot of the calculated first-order rate constant (k_3) for the dissociation of enzyme-inhibitor complex (E.I, CHT-A₄.TPCK) against pH. It was observed that in the alkaline region k_3 , which is calculated from the apparent first-order rate constant (k_{obs}) and K_I , remained relatively constant thus indicating that the decrease in k_{obs} was due to a decrease in the affinity (K_I) of the chymotrypsins for L-TPCK.

The ascending arms of the curves depicting the effect of pH on the rate of inhibition of CHT-A₄ with PMCK, BCK and β PECK strongly suggest the dependency of inhibition on a group possessing a $pK_a \sim 6.4$. Structural studies have confirmed the alkylation of the imidazole group of histidine-57.

Studies on the effect of pH on the inhibition of the

chymotrypsins with L-TPCK, PMCK and β PECK suggest that the pKa of the "active" histidine-57 in CHT-B may be lower than the corresponding residue in CHT-A₄.

The rate of inhibition of the chymotrypsins by L-TPCK was in agreement with earlier studies in this laboratory which indicated that CHT-B was more resistant to inhibition by di-phenylcarbamyl chloride (DPCC) and DFP than was CHT-A₄. Interestingly, this resistance to inhibition was not observed with PMCK, β PECK and BCK. In fact, CHT-B proved to be more susceptible to these reagents than CHT-A₄. These studies suggest that the two independent binding sites on the chymotrypsins, namely, the specific and the acylamido binding sites may not be identical in CHT-A₄ and CHT-B. Since PMCK, β PECK and BCK lack a substituent which will bind to the acylamido binding site, it has been suggested that this site in CHT-B likely accounts for the observed resistance of CHT-B to inhibition by L-TPCK, DFP and DPCC.

L-TPCK and PMCK appear to be true chymotryptic bifunctional reagents since they did not alter the activity of trypsin. Destruction of the tertiary structure of CHT-A₄ prevented the alkylation of both histidine and methionine. Furthermore, the inhibition of CHT-A₄ by these reagents was retarded in the presence of β -phenylpropionate, a competitive inhibitor, thus indicating that the bifunctional reagents were combining in a non-covalent manner with the active site

of the chymotrypsins prior to covalent attachment. Interestingly, L-TPCK and PMCK were found to be capable of alkylating sub-molar quantities of methionine when incubated with CHTG-A. This finding supports studies in the literature which suggest that the substrate binding site preexists in the zymogen.

CHT-A₄ and CHT-B were found to be irreversibly inhibited by TPCK and PMCK with the accompanying loss of 1.0 residues of histidine. Methionine sulfone analyses conducted on CHT-A₄-TPCK and -PMCK indicated the formation of approximately 0.4 residues of methionine sulfonium salt in the preparations. (CHT-B has not been investigated). Furthermore, analysis of CHT-A₄ incubated with D-TPCK revealed that only sub-molar quantities of methionine were alkylated.

Through application of the elegant diagonal paper ionophoresis technique developed by Dr. B.S. Hartley of the Laboratory of Molecular Biology, Cambridge, England, cysteic acid peptides containing methionine residues 180 and 192, both histidine residues (40 and 57), and the "active serine" residue (195) could be conveniently isolated from a peptic digest of native or alkylated chymotrypsins by first subjecting the digest to an initial ionophoresis at pH 6.5 and oxidizing a side strip of the ionogram with performic acid vapours. Re-running the oxidized band at right angles to the original ionophoresis, yielded the characteristic diagonal peptide map.

Those peptides not affected by performic acid were found to be on a line diagonally across the paper. However, those peptides which acquired a negatively charged cysteic

acid residue migrated off the diagonal in direction of the anode. Peptides were detected on the diagonal peptide map by using the cadmium-ninhydrin reagent and the specific Pauly reagent (diazotized sulfanilic acid) for histidine. Comparison of the native and TPCK- and PMCK- alkylated chymotrypsins revealed that the Pauly positive histidine-57 peptide on the diagonal peptide map of CHT-A₄ was replaced by a very acidic, Pauly negative histidine-57 peptide on the diagonal peptide maps of CHT-A₄-TPCK and -PMCK. Amino acid analyses of the acid hydrolysate of the performic acid oxidized histidine-57 peptide from L-TPCK and PMCK alkylated chymotrypsins were consistent with the known composition of the peptide except that histidine could not be detected. Instead, a new peak appeared in the vicinity of cystine on the amino acid analyzer. This peak has been positively identified as 3-carboxymethyl-histidine by comparison with the synthetically prepared derivative on the amino acid analyzer and by ionophoresis at pH 6.5. The formation of 3-carboxymethyl histidine in both PMCK- and TPCK- inhibited chymotrypsins was induced by a performic acid rearrangement of the ketone moiety of the histidine derivatives in which the phenoxymethyl group of the L-1-tosylamido-2-phenylethyl group migrates to form an ester. Acid hydrolysis of the ester yielded 3-carboxymethylhistidine. Thus, it may be concluded that the chymotrypsins are inhibited by L-TPCK and PMCK by virtue of the alkylation of histidine-57 at the nitrogen 3 position.

The particular methionine in CHT-A₄ modified by these

bifunctional reagents appears to be residue-192, three residues removed from the active serine residue-195, since a basic methionine sulfonium salt band was detected on the diagonal peptide map of CHT-A₄-PMCK migrating in advance of the native methionine-192 band.

From a comparison of L-TPCK, PMCK and EPOP it is possible to make certain conclusions concerning the relative importance of their structural features in determining the site of alkylation. First, the asymmetric carbon and the tosylamido group are unnecessary for alkylation of the histidine. However, if these are present, then the configuration must be of the L form for proper steric fit. Second, the replacement of the phenylmethylene group of the phenoxy radical is not effective in directing the alkylation to the methionine. Clearly, the major factor determining the site of attack is the nature of the alkylating group. Apparently the steric requirements for the approach of an alkylating group to the nitrogen 3 position of histidine-57 are sufficiently restrictive to make reaction between an epoxide and the imidazole nitrogen impermissible. The chloromethyl ketone derivatives meet these requirements, in whole or in part, and alkylation occurs.

α -Chloroacetophenone (CA, C₆H₅CO·CH₂Cl, n = 1) and a closely related reagent, anisoyl chloromethyl ketone (ACK, CH₃·O·C₆H₄·CO·CH₂Cl), have been shown by methionine sulfone analyses to alkylate one methionine residue in CHT-A₄. No alteration in the histidine was apparent. Diagonal peptide maps of CHT-A₄-

CA and CHT-A₄-ACK clearly revealed the presence of a methionine sulfonium salt of residue - 192. No alteration in methionine-180 was observed. The reduction in enzymic activity to 15% and 20% respectively was attributed to a marked decrease in the affinity of the modified enzyme for the substrate ATEE.

Amino acid analyses coupled with the methionine sulfone analyses indicated that benzyl chloromethyl ketone (BCK) and β -phenylethyl chloromethyl ketone alkylate both histidine (0.2 and 0.4 residues, respectively) and methionine (0.3 and 0.4 residues, respectively) in CHT-A₄. The residual activity of CHT-A₄ following inhibition with BCK and β PECK was found to be 35% and 2% respectively using a rate assay (ATEE).

Diagonal peptide maps of CHT-A₄-BCK and CHT-A₄- β PECK were found to be essentially identical and indicated that histidine-57 and methionine-192 were alkylated. Unlike the Pauly negative 3-carboxymethylhistidine-57 peptides isolated from L-TPCK and PMCK chymotrypsins, a Pauly positive alkylated histidine-57 peptide appeared on the diagonal. Amino acid analysis of this peptide isolated from CHT-A₄- β PECK failed to reveal histidine, but was otherwise identical to the native histidine-57 peptide. A qualitative amino acid analysis revealed a new Pauly positive amino acid migrating behind alanine during ionophoresis conducted at pH 1.8. The modified histidine-57 residue was suspected to be 2- (or 4-) hydroxymethylhistidine formed as a result of performic acid rearrangements of the ketone moiety of nitrogen-3 substituted.

β PECK-histidine or BCK-histidine derivative.

The loss in enzyme activity of CHT-A₄ following the alkylation with BCK and β PECK cannot be completely accounted for by the loss of histidine and methionine in amino acid and methionine sulfone analyses. It has been suggested that an additional group, possibly serine-195, is also alkylated by these reagents.

Based on the bifunctional reagents studied, requirements for histidine alkylation appear to be quite restrictive and depend on (1) the presence of a chloromethyl ketone group and (2) the exact spatial relationship between the chloromethyl ketone and the aromatic moiety. The precise relationship is attained by the presence of two methylene bridges (L-TPCK, β PECK) or a combination of an ether linkage and a methylene bridge (PMCK). Methionine alkylation, on the other hand, appears to be non-specific and occurs readily with a variety of reagents differing in the nature of the alkylating group and in the distance between the aromatic and alkylating group.

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List of Abbreviations

CHTG-A	Chymotrypsinogen A
CHT-A ₄	Chymotrypsin A ₄
CHT-A ₁	Chymotrypsin A ₁
CHTG-B	Chymotrypsinogen B
CHT-B	Chymotrypsin B
CHT-A ₄ -CA	Chymotrypsin A ₄ inhibited with α -chloroacetophenone.
CHT-A ₄ -ACK	Chymotrypsin A ₄ inhibited with anisoyl chloromethyl ketone.
CHT-A ₄ -BCK	Chymotrypsin A ₄ inhibited with benzyl chloromethyl ketone.
CHT-A ₄ - β SPECK	Chymotrypsin A ₄ inhibited with β -phenylethyl chloromethyl ketone.
CHT-A ₄ -TPCK	Chymotrypsin A ₄ inhibited with L-1-tosylamido-2-phenylethyl chloromethyl ketone.
CHT-A ₄ -PMCK	Chymotrypsin A ₄ inhibited with phenoxymethyl chloromethyl ketone.
CHT-B-TPCK	Chymotrypsin B inhibited with L-1-tosylamido-2-phenylethyl chloromethyl ketone.
CHT-B-PMCK	Chymotrypsin B inhibited with phenoxymethyl chloromethyl ketone.
DIP-CHT-A ₄	Diisopropylphospho-chymotrypsin A ₄ .
AE-CHTG-B	S-(β -aminoethyl)-chymotrypsinogen B.
L-TPCK	L-1-tosylamido-2-phenylethyl chloromethyl ketone.
D-TPCK	D-1-tosylamido-2-phenylethyl chloromethyl ketone.

N-methyl-L-TPCK	L-1-(N-methyl tosylamido)-2-phenylethyl chloromethyl ketone.
PMCK	Phenoxymethyl chloromethyl ketone.
β PECK	β -phenylethyl chloromethyl ketone.
BCK	Benzyl chloromethyl ketone.
CA	α -chloroacetophenone
ACK	Anisoyl chloromethyl ketone
EPOP	1,2-epoxy-3-phenoxypropane
DFP	Diisopropylphosphofluoridate
FDNB	Fluorodinitrobenzene
DPCC	Diphenylcarbamyl chloride
PMSF	Phenylmethane sulfonyl fluoride
ATEE	N-Acetyl-L-tyrosine ethyl ester
ATME	N-Acetyl-L-tyrosine methyl ester
APEE	N-Acetyl-L-phenylalanine ethyl ester
ATryEE	N-Acetyl-L-tryptophan ethyl ester
AE-cysteine	S-(β -aminoethyl)-L-cysteine
PITC	Phenylisothiocyanate
TFA	Trifluoroacetic acid
DNS-Cl	1-dimethylaminonaphthalene-5-sulphonyl chloride (Dansyl chloride)
DNS-AA	Dansyl amino acid
DEAE-cellulose	Diethylaminoethyl-cellulose
K_m	Michaelis constant
K_I	Equilibrium constant for enzyme-inhibitor complex
tris	tris(hydroxymethyl)amino methane
3-CM histidine	3-carboxymethylhistidine

I. INTRODUCTION

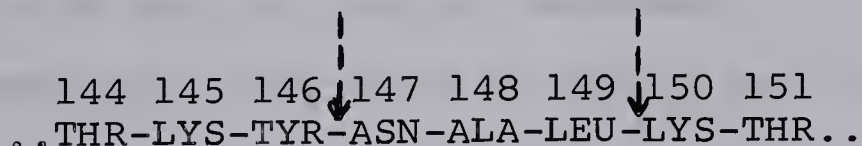
During the past decade, a great deal of attention has been directed to the study of proteins isolated from the bovine pancreas gland. The most definitive investigations have centered on insulin, ribonuclease, trypsinogen and chymotrypsinogen A (CHTG-A). CHTG-A was first crystallized by Northrop and Kunitz in 1930 (1), and its availability in recent years has stimulated much of the research directed towards it. Lacking the presence of a prosthetic group or cofactor, the biological activity of chymotrypsin A₄ (CHT-A₄) is vested solely in the amino acid sequence and the unique tertiary structure of the enzyme.

The rapid activation of CHTG-A by trypsin, enterokinase or pronase, has been demonstrated to proceed through cleavage of an arginine-isoleucine bond (residues 15 and 16) to yield the most active form of chymotrypsin-CHT-A₁ (8-10). Chymotryptic cleavages at leucine-13, to liberate a basic dipeptide serinyl-arginine, and at tyrosine-146 and asparagine-148 to liberate a neutral dipeptide threoninyl-asparagine, yielded CHT-A₂ and the common CHT-A₄, respectively. CHT-A₄ consists of three chains designated A, B and C linked covalently by disulfide bridges. The short A chain (13 residues) is linked to the B chain (130 residues) by a disulfide bridge from the N-terminal half-cystine to position-122. Only one interchain disulfide

bridge joins the B and C chains. Intrachain disulfide bridges form the structurally important histidine loop and methionine loop in the B and C chains respectively. One additional intrachain disulfide bridge in the C chain in conjunction with the methionine loop disulfide bridge and the interchain disulfide bridge between the B and C chains form what has been referred to as the "serine knot" in proximity to the active serine residue (11,12).

An isozyme of CHTG-A, CHTG-B, was first isolated by Laskowski in 1946 (2). Crystallization was achieved two years later by Brown, Shupe and Laskowski (3). Contrary to earlier reports (4), CHTG-B was found to be present in amounts equal to CHTG-A (16% by weight of the bovine pancreatic juice) (5). Similarities in specificity between CHT-A₄ and CHT-B have been shown to exist (6,7). Only recently has the knowledge of the structures CHTG-B and CHT-B been extended to permit comparisons with the corresponding A proteins (14,17).

Activation of CHTG-B by trypsin is concomitant with the hydrolysis of arginine-15 isoleucine-16 bond to yield CHT-B₁ (13). Subsequent chymotryptic autolyses appear to occur in the vicinity of residue 146 (13,14), and are confined to the sequence:



N- and C-terminal analyses of the isolated B and C chains as well as the isolation and sequencing of tryptic and chymotryptic peptides from these chains has confirmed these sites of

chymotryptic autolyses in chymotrypsin B. The absence of an activation peptide from the N-terminal region (residues 14 and 15) has been attributed to the replacement of serine-14 by alanine in CHTG-B (14,15).

From tryptic and chymotryptic digests of reduced and carboxymethylated chymotrypsinogen B, of the reduced and aminoethylated zymogen and of the separated B and C chains of the reduced and carboxymethylated enzyme, it has been possible to isolate and sequence peptides accounting for the entire structure. On the basis of homologous sequences in chymotrypsinogen A, these peptides were arranged to facilitate a comparison of the structures of the two proteins. Final proof of the sequence has been recently obtained through the elucidation of appropriate overlap peptides. Of the 245 residues present, approximately 54 are different. Eliminating different but chemically similar substitutions, for example, glutamic and aspartic acids, this number is reduced to 35. The majority of these differences are located as repeating groups in the B chain from residues 77 to 149. A concentrated region of amino acid differences occurs at the points of chymotryptic autolysis between the B and C chains. Of the total number of differences, approximately 40% involve charged residues.

The homologous structures in CHTG-A and CHTG-B have supported physical studies which have compared the hydrodynamic and optical rotatory dispersion properties of the two proteins. Similar tertiary structures of the zymogens are indicated by almost identical sedimentation and viscosity parameters and by

estimates of similar low helical content (20). Spectral changes accompanying the activation suggest only minor conformational alterations and possibly reflect the perturbation of a tyrosine residue (147). The decrease in levorotation, as shown in the Moffitt equation as a difference in a_0 , is felt to be concomitant with the orientation of histidine-57 and serine-195 into juxtaposition in the active sites of the enzymes. Substantial evidence has been obtained for CHTG-A to suggest the preexistence of the specific binding site (18,19) in the inactive zymogen.

Sequence studies have revealed that both CHTG-A and CHTG-B probably consist of a single polypeptide chain of 245 residues (11,14). These figures correspond to a molecular weight of approximately 25,000 determined by physical techniques (20).

The marked contrast in the isoelectric point of the zymogens (CHTG-A, 9.5 (21); CHTG-B, 5.2 (13)) can be related to the distribution of amide, acidic and basic groups. Interestingly, the sum of acidic and amide groups in the zymogens is identical. Deamidation of eight amides and the replacement of two basic groups with neutral amino acids accounts for the acidic isoelectric point of CHTG-B (20).

The release of the basic dipeptide SER-ARG during the formation of CHT-A₄ is responsible for the decrease in the isoelectric point to 8.0. CHT-B, on the other hand, has an isoelectric point only 0.2 pH units below that of CHTG-B. This observation is in accord with the loss of sub-molar neutral peptides and may be attributed to the perturbation of an acidic residue.

The specificity of CHT-B towards aromatic amino acids resembles that of CHT-A₄. A distinguishing feature of CHT-B appears to be the facility with which leucine bonds are cleaved (6). Both chymotrypsins are capable of hydrolyzing peptide bonds in a manner not entirely consistent with their activity towards synthetic substrates (22,23). The reason for non-specific cleavages undoubtedly resides in the environment of the susceptible bond. The inherent tryptic activity of the chymotrypsins is a true property of the enzymes and not a manifestation of tryptic contamination.

Although CHTG-B is activated at a rate six times greater than is CHTG-A, under rapid activation conditions, the maximum activity towards N-acetyl-L-tyrosine ethyl ester (ATEE) was found to be lower (13). The zero order rate constant expressed as k' (meq substrate/ml hydrolyzed per min. per mg enzyme nitrogen/ml) was found to be 4.6 for CHT-A₄ and 3.3 for CHT-B (129).

A comparison of the second order rate constants for the reaction of diisopropylphosphofluoridate (DFP) with CHT-A₄ ($317 \ell \text{ moles}^{-1} \text{ sec}^{-1}$ (24)) and CHT-B ($35 \ell \text{ moles}^{-1} \text{ sec}^{-1}$ (13)) reveals the resistance of the latter to inhibition. By analogy, diphenylcarbamyldichloride (DPCC) and L-1-tosylamido-2-phenylethyl chloromethyl ketone (L-TPCK) inactivate CHT-B at a rate 5-fold and 2.5-fold less, respectively, than CHT-A₄. Contrary to this trend, phenoxymethyl chloromethyl ketone (PMCK), β -phenylethyl chloromethyl ketone (β PECK) and benzyl chloromethyl ketone (BCK) inactivate the chymotrypsins at comparable rates (Chapter III). The rates of deacylation of trimethyl-

acetyl-CHT-B, cinnamoyl-CHT-B, and anisoyl-CHT-B exceeded those of the corresponding acyl-intermediates of CHT-A₄. These observations must reflect subtle environmental differences in the active sites of the enzymes.

Much work has been directed towards elucidating the nature of the active site of CHT-A₄. CHT-B has hitherto only recently received attention. The discussions to be presented are confined for the most part to CHT-A₄, but a parallel undoubtedly exists with CHT-B albeit experimental evidence is limited.

Studies of CHT-A₄ applying diversified reagents and techniques have implicated the presence of serine, histidine, methionine, tryptophan and isoleucine at or near the active site. The inhibition of CHT-A₄ by the nerve gas DFP was first reported by Jansen, Nutting and Balls in 1948 (25). The inhibition was demonstrated to be stoichiometric (26) while the isolation of phosphoserine from DIP-CHT-A₄ supported the acylation of a serine residue (27). The particular serine residue involved was identified in the sequence GLY-ASP-SER-GLY through partial acid or enzymic hydrolysis of DI P-CHT-A₄ (28-31). Similar sequences have been identified at the active sites of several of the "serine" enzymes which are distinguished by their ability to react with DFP (32).

Selective modification of residues directly associated with the active site of CHT-A₄ or in proximity to it has yielded considerable information concerning their involvement in catalysis. Compounds which have received the most attention

have been referred to as bifunctional reagents, substrate analogue reagents, or pseudosubstrates. The term "bifunctional" denotes a molecule endowed with two separate functional groups differing in their mode of attachment to the enzyme. A common structural feature of chymotryptic reagents is the presence of an aromatic group which is capable of mimicking a substrate molecule and thereby being bound to the enzyme surface. Covalent attachment of the bound reagent is facilitated by a reactive moiety as the second functional group. To date the reactive moieties exploited have included chloromethyl ketones, sulfonyl chlorides and fluorides, acyl chlorides, epoxides, tosyl and p-nitrophenol reagents.

The term "bifunctional reagent" has also been used in connection with reagents possessing two groups capable of reacting covalently with a protein molecule. Zahn pioneered much of the work on this type of reagent. Studies on the Zahn reagents, p-p'-difluoro-m,m'-dinitro-diphenyl sulfone by Wold (113) and 1,5-difluoro-2,4-dinitrobenzene by Marfey et al. (110-112), have illustrated the use of these reagents in establishing intramolecular crosslinkages between lysine and/or tyrosine residues. Recently diimido adipimide (Cl H₂N : C(OCH₃) . [CH₂]₄ . C(OCH₃) : NH₂Cl) has been suggested for the elucidation of the three dimensional localization of amino acid residues in proteins (124).

Diphenylcarbamyl chloride is capable of inactivating CHT-A₄ at a rate twice that of DFP (33). The ease with which

inactivation occurs is attributed to the tenacious binding of the phenyl rings to two independent, predominately hydrophobic, sites; the acylamido and the specific binding site (34-36). Considering a general substrate $R_1\text{.CONH.CH(R}_2\text{).C(=O)X}$, the acylamido binding site accommodates the $R_1\text{.CONH-}$ substituent through a combination of hydrogen and hydrophobic bonds (35, 37, 38, 39). The purely hydrophobic nature of the specific binding site accommodates the R_2 substituent upon which specificity is dependent. The fact that DPCC is bound to CHT-A₄ via both aromatic rings, is shown by the recent finding that N,N-methyl-phenyl-carbamyl chloride is an inferior inactivator by a factor of approximately 150 (40). Erlanger and Edel (41) have utilized the inherent binding capabilities of the diphenylcarbamyl moiety in the development of an all-or-none assay technique using 2-nitro-4-carboxyphenyl-N,N-diphenyl carbamate as a chromogenic inactivator. Inhibition of CHT-A₄ by DPCC is presumably due to the acylation of a serine residue (42,43). The reactivation of DPC-CHT-A₄ following exposure to nucleophiles such as isonitrosoacetone and hydroxamic acid derivatives supports this view (33).

Kallos (44,45) has studied the effect of replacing the ester moiety of a phenylalanine substrate with an alkyl sulfonylated primary alcoholic group - $\text{C}_6\text{H}_5\text{.CH}_2\text{.CH(NH.R') .CH}_2\text{O.R}$. Substrate analogue reagents—L- and D-phenylalaninol ditosylate (R and R'= $\text{SO}_2\text{C}_7\text{H}_7$); L- and D-N-acetyl-phenylalaninol methyl sulfonate (R= SO_2CH_3 and R'= COCH_3)—led to the alkylation of the active site of chymotrypsin presumably through a nucleophilic

attack on the alcoholic carbon by the active serine residue. The suitability of the aryl sulfonyl moiety as a leaving group was in accord with this postulate. It has been observed that the L-isomers inactivate CHT-A₄ at a rate 3-fold greater than the D-isomers. The mode of inactivation, particularly of the D-isomers, has not been reported to date.

Perhaps one of the most interesting reagents has been advanced by Lawson and Schramm (46-48). p-Nitrophenyl bromoacetyl- α -aminoisobutyrate readily forms an inactive acyl-CHT-A₄ with the simultaneous release of p-nitrophenol. The reagent had no noticeable effect on trypsin, CHTG-A or DIP-CHT-A₄. Incubation of the bromoacetyl- α -aminoisobutyl-chymotrypsin at low pH favors an intramolecular alkylation of methionine-192. Following hydrolysis of the acyl-enzyme bond, an irreversibly modified chymotrypsin possessing about 20% residual activity was formed. The decrease in activity was associated with a 10-fold increase in the Michaelis constant. DFP was still capable of reacting stoichiometrically with the modified enzyme.

A similar approach to enzyme modification has been briefly reported by Gold (49). Trypsin was rapidly phosphorylated by ethyl-5-iodopentyl phosphonofluoride. Subsequent incubation of the iodoalkyl-phosphoryl trypsin at pH 8 led to the loss of the iodine atom through an intramolecular alkylation with a residue as yet unidentified. The possibility of intermolecular alkylation to form a trypsin dimer has been refuted. Chymotrypsin was rapidly phosphorylated by the reagent but loss

of the iodine atom was much slower.

Gundlach and Turba (153) attempted to modify CHT-A₄ by incorporating chloroacetyl and iodoacetyl substituents into substrate and inhibitor molecules. Incubating N-iodoacetyl-D-phenylalanine methyl ester with CHT-A₄ at pH 7 resulted in the loss of 50% of the enzyme activity and was shown to be associated with the modification of a methionine residue. The L-isomer of the foregoing compound decreased the activity of CHT-A₄ only slightly, whereas the corresponding D and L chloroacetyl derivatives, iodoacetate and iodoacetamide had no observable effect.

Westheimer et al. (114,115) have developed a novel method by which an extremely reactive moiety could be generated in the active site of an enzyme. Acylation of CHT-A₄ with p-nitrophenyl diazoacetate yielded, as the acyl-intermediate, diazoacetyl-CHT-A₄. Subsequent photolysis led to the decomposition of the diazo group with formation of a reactive carbene (E.CH₂.O.CO.CH:). Although regeneration of enzymatic activity and the production of glycolic acid was the predominant course of the photolysis, O-carboxymethylserine, O-carboxymethyltyrosine and l-carboxymethylhistidine were isolated from acid hydrolysates of the photolysed acyl-enzyme.

Numerous studies conducted with derivatives of sulfonyl chlorides and fluorides have demonstrated unequivocally the essential role of serine in chymotryptic catalysis. Strumeyer, White and Koshland (50) inactivated CHT-A₄ with p-toluenesulfonyl (tosyl) chloride—a reagent known to selectively acylate serine-

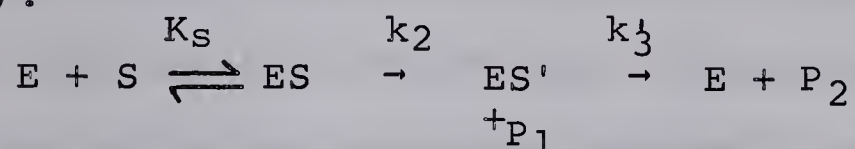
195 (51). Treatment of tosyl-CHT-A₄ with dilute base resulted in the elimination of the tosyl group with the formation of an α,β -double bond. The anhydrochymotrypsin so formed was devoid of enzymatic activity thus dispelling the belief that serine was acting as a scavenger in acyl-enzyme formation (127,154-155). Since the dehydroalanine residue formed had less "bulk" than the serine residue, the steric access arguments purporting that the acylated serine sterically hindered the approach of substrate to the true catalytic group, histidine, were invalidated (142).

Heavy atom labelling of the active site of CHT-A₄ has been accomplished with pipsyl chloride ($\text{IC}_6\text{H}_4\text{SO}_2\text{Cl}$) (51) and p-nitrophenyl-2-phenyl-2-methoxy-1-mercuryl chloride propionate (52). The former reagent is known to acylate the serine hydroxyl while the latter is presumed to act in a similar manner. These reagents have potential value in crystallography studies particularly for an enzyme of known sequence.

A series of sulfonyl fluorides capable of inactivating CHT-A₄, trypsin and, to a certain extent, acetylcholinesterase have been reported by Fahrney and Gold (53). The most potent tryptic and chymotryptic inactivator was found to be phenylmethanesulfonyl fluoride (PMSF). An increase or decrease of one methylene group separating the phenyl and sulfonyl fluoride moieties resulted in a reduction in the effectiveness of the reagent. PMSF has been shown to react stoichiometrically with a serine residue in CHT-A₄ (54,43); the rate of inhibition being comparable to DFP (53). The markedly lower vapour pressure of PMSF is an inherent safety feature when compared to the toxic

nerve gas DFP.

Many of the reagents discussed to date inactivate chymotrypsin in an irreversible manner; the deacylation rate of the acyl-CHT-A₄ being essentially zero in the absence of supplementary nucleophiles. An acyl-enzyme mechanism, of the form shown below, was first proposed by Hartley and Kilby (55) and has been supported by extensive studies recently reviewed by Bender et al. (56,57).



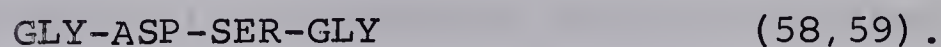
where: ES is the enzyme-substrate complex

ES' is the acyl-enzyme

P₁ is the leaving group

and P₂ is the carboxylate ion product.

The form presented for the chymotryptic mechanism was first suggested by the reaction with p-nitrophenylacetate (NPA) (55). A rapid "burst" of p-nitrophenol (P₁) followed by a slow linear release was indicative of an extremely fast acylation of enzyme (ES') followed by a rate determining deacylation reaction (i.e. $k_2 \gg k_3$). The acetyl-CHT-A₄ so formed as the acyl-intermediate has been isolated at low pH and the acetyl group was found to reside on the serine residue in the sequence:



Analogous acyl-chymotrypsins were conveniently prepared from the p-nitrophenyl esters of isobutyrate, trimethylacetate, hydrocinnamate and hippurate (60). A study of the deacylation rates revealed the stability of branched aliphatic acyl-CHT-A₄

derivatives in contrast to the lability of acyl-CHT-A₄ containing aromatic side chains.

Liberation of p-nitrophenol during the rapid, stoichiometric acylation of CHT-A₄ by NPA provided a suitable method to determine the concentration of active enzyme. Adoption of this technique for enzyme assays pioneered the development of what is referred to as "all-or-none" assays. Schonbaum, Bender and Zerner (61) were first to employ imidazolium compounds to an "all-or-none" assay system for CHT-A₄. Cinnamoylimidazole (CI) was chosen since acylation proceeded extremely rapidly under conditions where the deacylation of the cinnamoyl-CHT-A₄ and the spontaneous hydrolysis of CI were relatively slow. The absorbance maxima of CI and cinnamoyl-CHT-A₄ were sufficiently separated so as to allow for determination of the enzyme concentration from the decrease in CI absorbance at 335 mμ or 310 mμ. Evidence suggests that the moiety acylated was the hydroxy group of serine (62).

Although much of the work with CHT-A₄ has involved the active serine residue, other amino acids, particularly tryptophan, isoleucine, methionine, and histidine, have been implicated in the biological expression of the enzyme. Oxidation of CHT-A₄ by horseradish peroxidase and hydrogen peroxide caused a 50% reduction in esterolytic activity as measured against tyrosine ethyl ester. The oxidation of a tryptophan residue was suspected to be associated with the decrease in activity since no change in the histidine or tyrosine content of the enzyme occurred (63). Formation of methionine sulfoxide, however, could have partially

contributed to the loss in activity. Hachimori et al. (64,145) inactivated CHT-A₄ to 15% of the original activity by oxidizing three tryptophan residues with a mixture of hydrogen peroxide and dioxane at pH 8.4. The state of other residues, particularly methionine and histidine, was not reported. The observation that two hydrogen peroxide sensitive tryptophan residues in CHT-A₄ were transformed into non-oxidizable residues following inhibition of the enzyme with DFP was of interest. Several studies have shown that absorption of substrate or DFP to the binding site of CHT-A₄ causes spectral changes associated with the "burying" of tryptophan residue(s) (65-68).

Neurath and Hartley (69) have postulated the presence of a hydrophobic slit with a narrow neck formed by two tryptophan residues in the binding site of CHT-A₄. Support for their theory was gleaned from studies with a β -substituted substrate, N-acetyl- $\beta\beta'$ -dimethyl-L-tyrosine methyl ester, which was found to be completely resistant to chymotryptic attack (70). The K_m was found to be markedly increased when compared to N-acetyl-L-tyrosine methyl ester (ATME). Replacement of the α -hydrogen in ATME or N-acetyl-D-tyrosine, with a methyl group, alters the K_m or K_I less than an order of magnitude, but, for the substrate, ATME, such a replacement decreases the rate of formation of products by a factor of 10^5 (71). Thus, an α -alkyl substituent does not suppress the binding (K_m) of a substrate to the extent that a β -substituent does, but has a drastic effect on the overall rate of catalysis. Comparison of N-acetyl-L-leucine and N-acetyl-L-valine methyl esters indicates that the presence of

an unsubstituted β -methylene group causes a 36-fold decrease in K_m while enhancing the acylation rate constant, k_2 , approximately 30-fold (72).

Recently, a highly selective reagent, 2-hydroxy-5-nitrobenzyl bromide (\emptyset' -Br), capable of alkylating approximately two tryptophan residues in CHT-A₄, at pH 3, has been reported (73-75). The reagent possesses a half-life in water of less than 30 seconds at pH 5, 25°, and appears to be 10^4 times more reactive towards tryptophan than water. The residual activity of the modified CHT-A₄ was, unfortunately, not reported. \emptyset' -Br is capable of reacting with methionine but the sulfonium salt so formed rapidly decomposes with the regeneration of the amino acid. When the reagent was incubated with a mixture of amino acids at pH 11, only tyrosine and cysteine, in addition to tryptophan, could successfully compete with water and OH⁻ ions for the reagent. Histidine did not react.

The presence of tryptophan residues in the active site was further supported by the studies of Dixon and Schachter (76). The rapid inactivation of CHT-A₄ in the presence of ATEE, or other specific substrates, and hydrogen peroxide, at pH 3.0, was attributed to the oxidation of two methionines, one to two residues of half-cystine and one to two residues of tryptophan. Nucleophilic attack by hydrogen peroxide on the acyl-enzyme was suspected to yield a peracid which, being momentarily present in the active site, could oxidize the susceptible residues.

Although the occurrence of lysine and tyrosine has been established in the active site of aldolases (77) and carboxypep-

tidase (78), respectively, their role in chymotrypsin appears inconsequential. Lysine residues can be chemically modified without affecting the activation of CHTG-A nor the activity of CHT-A₄ (79). Studies by Filmer and Koshland (80) and Dube, Roholt and Pressman (81), although contradictory in some respects, suggest that tyrosine is not directly involved in the active site, but may well tend to stabilize the tertiary structure. Chymotryptic activity was unaltered by the introduction of four iodine atoms, but as the extent of iodination increased to 6.3 atoms, the K_m (ATEE) increased 6-fold without any change in the V_{max} (80). Exhaustive iodination led to disruption of the tertiary structure and to modification of histidine, methionine, and tryptophan residues. The tyrosine most rapidly iodinated has been identified as the C-terminal residue of the B-chain (82,83).

An acidic group of pK_a 8.5, seen solely during acylation, appears to control the activity and conformation of chymotrypsin. The group has tentatively been identified as the α -amino group of isoleucine-16, the N-terminal residue of the B-chain (84). Acetylated-CHTG-A in which the N-terminal half-cystine, the ϵ -amino groups of lysine and two out of four tyrosines had been acetylated, was activated by trypsin in the presence of indole to yield an active species, acetylated-CHT-A₁, possessing only the free amino group of isoleucine-16. Further acetylation of N-terminal isoleucine-16 inactivated the enzyme. A comparative study of acetylated CHTG-A, CHT-A₁ and DIP-CHT-A₁, revealed the presence of two, three and two titratable groups respectively,

in the pH region 6.5 to 10. The common groups titrated in all three preparations were two histidine residues. The additional titratable group ($pK_a \sim 8.3$) in acetylated-CHT-A₁, which was, interestingly, absent in acetylated-DIP-CHT-A₁, has been associated with the α -amino group of isoleucine. Additional support was obtained from studies of the pH-dependent specific rotation $[\alpha]_\lambda$ of CHT-A₁ and acetylated-CHT-A₁ in the pH region 7 to 11 (67, 126). The rotational change in both cases accompanied the dissociation of a group with $pK_a \sim 8.5$. The specific rotation of acetylated-CHT-A₁ at pH 7.0, was essentially identical to the specific rotation of acetylated-DIP-CHT-A₁ at pH 10, thus strongly suggesting that a protonated form existed in both modified enzymes (85-87).

A primary amino group in the active site of trypsin has been demonstrated by Hofmann and Scrimger (148) to be indispensable in the biological expression of the enzyme. The rapid inactivation of trypsin by sodium nitrite at 0°, below pH 4.5, was concomitant with the loss of the incorporation of $DF^{32}P$ and was associated with the loss of about one lysine residue and the N-terminal isoleucine residue in addition to limited destruction of tryptophan. The trypsin substrate, benzoyl arginine ethyl ester, but not the chymotrypsin substrate, ATEE, protected the enzyme against sodium nitrite inactivation. The potential activity of trypsinogen treated with sodium nitrite was only slightly affected. On the basis of the pH dependency of the inactivation of trypsin, the electrostatic interaction of the primary amino group of isoleucine with a carboxyl group was suggested.

Substantial evidence exists associating the thioether side chain of methionine-192 with the hydrophobic substrate binding site (R_2) on the enzyme surface. Methionine-192 can be readily transformed into its sulfoxide derivative through photooxidation (80,88,89,156), or upon exposure to hydrogen peroxide (76,90,156) or NaIO_4 (91,92). (Methionine-180, on the other hand, remains unaltered and has been designated as the "buried" methionine. It is this methionine which is involved in the "methionine loop" of the chymotrypsins (12).) The resultant modified enzyme shows a 3-fold increase in the K_m for the substrate ATEE while no alteration in the V_{max} was observed. Rate (or efficiency) assays indicate that only 37% of the original activity was present whereas all-or-none assays using cinnamoylimidazole (88) showed the enzyme to be fully active. The insertion of a hydrophilic methionine sulfoxide side chain into a hydrophobic region was thought to lead to a partial disorganization of the substrate binding site.

Brown and Hartley (42,93) reported the stoichiometric inactivation of CHT- A_4 by a series of 1,2-epoxy-3-alkoxypropanes where the alkoxy group was phenoxy, o-nitrophenoxy or α -naphthoxy. These bifunctional reagents led to the S-alkylation of methionine-192 likely through the secondary carbon of the epoxy ring.

The existence of a histidine residue in the active site of chymotrypsin has been repeatedly implicated, but evidence, until recent years has been circumstantial. Based on pH-activity data and dissociation energy of ATEE and N-acetyl-L-

tryptophan ethyl ester (ATryEE), Cunningham (94) and Cunningham and Brown (95) have shown catalysis of the esters to be dependent on a group with pKa 6.71 and a ΔH of ionization of 11 kcal/mole. Hammond and Gutfreund (96) obtained a similar pKa value (6.85) from the hydrolysis of N-acetyl-L-phenyl-alanine ethyl ester (APEE) by CHT-A₄. The data suggested the involvement of the imidazole side chain of histidine but was equivocal.

Evidence derived from photooxidation studies also intimated the presence of histidine. In 1953, Wiel et al. (97) demonstrated that the inactivation of CHT-A₄ by photooxidation could be ascribed to the oxidation of one of the two histidines and three of the seven tryptophan residues. A reexamination of CHT-A₄ photooxidation under different conditions by Koshland, Strumeyer and Ray (88) revealed the modification of only a methionine and a histidine residue. The rate at which a histidine was destroyed correlated well with the reduction in enzymic activity as shown by the all-or-none assay.

Until the recent development of specific bifunctional reagents, alkylation of a histidine with 1-fluoro-2,4-dinitrobenzene (FDNB) was the first study to associate loss of activity with a histidine modification (98). Close agreement existed between the mole fraction of histidine reacting with FDNB and the loss of esterase activity and DFP binding ability. The incorporation of approximately 2.0 moles of FDNB per mole of CHTG-A did not alter the histidine content nor did it affect subsequent activation by trypsin. Unfortunately, the alkylation of groups in addition to histidine in CHT-A₄ reduced the significance of the findings.

Labelling of the active site of CHT-A₄ with the fluorescent dye, 1-dimethylaminonaphthalene-5-sulfonylchloride (Dansyl chloride), and comparing the fluorescent color to dansylated amino acids, led Hartley and Massey (99) to postulate the alkylation of a histidine residue. Gundlach, Kochnec and Turba (100) supported this interpretation through studies on diazotized, dansylated CHT-A₄. Coupling of diazobenzene sulfonate was assumed to occur readily with free histidine but not with dansyl histidine. Subsequent acid hydrolysis of the bis-derivatized CHT-A₄ yielded free histidine from the labile dansyl histidine; diazotized histidine was stable under these conditions. The low recovery of histidine (0.3 residues instead of approximately one residue) was attributed to the alkaline instability of dansyl histidine in CHT-A₄ during diazotization. The free histidine, formed from the decomposition of dansyl histidine, was rapidly diazotized thus resulting in a low dansyl histidine content prior to acid hydrolysis. The lability of dansyl histidine at alkaline pH has been disputed by Staab and Wendel (125) through studies on N-benzene sulfonylimidazole. Discussions on the nature of diazo coupling to the imidazole nucleus presented by Hofmann (126) are not in accordance with the assumptions of Gundlach et al. (100). Recent studies by Gold (43) strongly suggest that dansyl chloride inhibits CHT-A₄ by sulfonylating the active serine residue. Treatment of various CHT-A₄ derivatives (DIP-, Dansyl-, PMSF-) with 2-mercaptoethylamine led to the formation of S-(β -aminoethyl)cysteine via displacement reactions. The existence of dansyl histidine

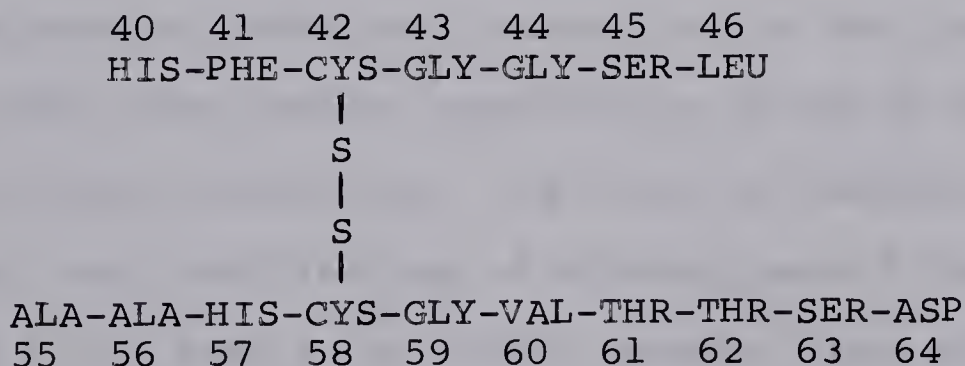
was thought to be the result of nonspecific sulfonylation.

Conclusive evidence for the involvement of a histidine residue in the active site of CHT-A₄ remained quite elusive until a highly selective reagent, L-1-tosylamido-2-phenylethyl chloromethyl ketone (L-TPCK) was found to be an irreversible inhibitor of CHT-A₄ (101,102). The inhibition was found to be associated with the loss of one of the two histidine residues upon amino acid analysis (103,104).

When the writer began his research in 1962, the work in this laboratory was largely directed towards the characterization of the CHTG-B zymogen and its active enzyme. At this time, almost nothing was known of the primary structure of the enzyme nor of the residues involved in its catalytic activity. The description at that time by Schoellman and Shaw (101,102), of the inactivation of CHT-A₄ by the bifunctional reagent, L-TPCK, offered an opportunity for the investigation of the role of histidines in the catalytic activity of CHT-B. Our initial endeavour, then, was the synthesis of this reagent and a study of its reaction with CHT-B. It was soon shown that like CHT-A₄, the irreversible inhibition of the B enzyme was accompanied by the loss of one of the two histidine residues present in the molecule. Attempts were then initiated to isolate and characterize the modified histidine as a peptide from proteolytic digests of the inhibited enzyme.

At this point, the development of the "diagonal" electrophoretic technique was brought to our attention by Dr. B. S. Hartley of the Laboratory of Molecular Biology at

Cambridge, England. By this method, it was possible to isolate, in a relatively simple manner, sequences about the disulfide bonds of proteins as pairs of cysteic acid peptides from peptic digests of the native proteins. Utilizing this technique, Brown and Hartley (93,132) were soon successful in elucidating the disulfide bridges of CHTG-A. Significantly, the cysteic acid peptides of CHTG-A isolated in this way are of particular interest since they include those parts of the primary structure known to be involved in the catalytic activity of the enzyme. In particular, peptides containing both of the histidines, residues 40 and 57, serine-195 and methionine-192 are easily purified in this way. The development of this procedure soon led to the identification of histidine-57 of CHT-A₄ as the site of alkylation by L-TPCK by Smillie and Hartley (105), and of the elucidation of the disulfide bridges of CHTG-B by the same workers (17). This latter work further demonstrated that the two histidines in CHTG-B were linked through a disulfide bridge and that this structure had an identical amino acid sequence to that in CHTG-A.



It was clear from these studies that the site of alkylation of CHT-B by L-TPCK was readily amenable to elucidation by these techniques and it was soon demonstrated to be identical

with the same residue in CHT-A₄, that is, histidine-57 (see Chapter IV).

From the work of Smillie and Hartley (16,17), it became clear that the structure involving two histidines brought close to each other by a cystine bridge was common not only to the two chymotrypsins but to trypsin and elastase as well. The elucidation of the complete amino acid sequences of chymotrypsinogen A by Hartley (11) and of trypsinogen by Walsh et al. (149,150) demonstrated that the near-identity of the sequence in the region of half-cystine-42 and preceding half-cystine-58 extends to the complete sequence between these two half-cystines in what may be called a "histidine-loop".

The importance of this "histidine-loop" structure as a possible common hydrophobic substrate binding site in the two enzymes has been discussed by Bender, Killheffer and Kezdy (152). This postulate is supported by the observation that the rates of deacylation of a number of non-ionic acyl-chymotrypsin A₄ and acyl-trypsin derivatives are essentially identical over a 10⁵-fold range of rate constants (151). This kinetic identity must reflect an identical interaction of the various acyl-groups with very similar specificity sites of both enzymes.

In this laboratory, the rates of deacylation of several non-ionic acyl-derivatives of chymotrypsin B have been demonstrated to be some 4- to 5-fold greater than the corresponding rates for CHT-A₄ (13). These observations immediately raised the question as to the nature of the sequence of that portion of the "histidine-loop" of chymotrypsin B which had not been

isolated by the diagonal procedure; namely, the sequence corresponding to residues 47 to 54 for chymotrypsin A₄. For this reason, this worker undertook to extend the sequence analyses of the "histidine-loop" of CHT-B and the results of this study are presented in Chapter II of this thesis.

Chapters III and IV of this thesis are concerned with the rates and site of reaction of several aromatic chloromethyl ketone derivatives with the chymotrypsins. These studies were prompted by the findings in several laboratories (42,48,104-108) that the reagent L-TPCK reacts apparently specifically with histidine-57 of CHT-A₄ whereas a number of other similar bifunctional reagents alkylate methionine-192. In particular, the work of Brown and Hartley (42) demonstrated that 1,2-epoxy-3-phenoxypropane (EPOP) led to the inhibition of CHT-A₄ by the specific alkylation of methionine-192. Because of the similarities in the structures of this compound and L-TPCK, it was of considerable interest to determine what structural features directed their point of attack to one residue or another at the active site of chymotrypsin. For this purpose, a series of aromatic chloromethyl ketone derivatives related to EPOP and L-TPCK were synthesized and their inhibition of chymotrypsin was studied.

II. SEQUENCE OF THE "HISTIDINE-LOOP" IN CHYMOTRYPSINOGEN B

1. Introduction

Studies of the structures of similar proteins from different species have led to the elucidation of striking homologies in amino acid sequence. One may reasonably postulate the possibility of evolutionary precursors possessing similar essential structural features for biological activity. A process of divergent genetic alteration has been advanced to account for structural homologies present in haemoglobin (116-118), cytochrome c (119-121), and ribonuclease (122). Sanger (123), through a study of bovine, porcine, sheep, equine and whale insulins, showed the only discrepancies in amino acid sequence to occur between the intrachain disulfide bridge in the A chain. It was suggested that this region, consisting of only three amino acids, was not required for biological activity. Structural homologies between yeast and horse heart cytochrome c have been presented by Horinishi et al. (128). Identical positioning of three histidine residues relative to the heme-linked cysteine residues has implicated these structures as being essential for biological activity, showing a remarkable preservation from bacterial through to mammalian systems.

In general, homologous regions in proteins represent structures which are likely to be an absolute requirement for biological activity, and thus have been preserved through successive mutations. A mutation in these essential structures

would be considered lethal, thus terminating the unproductive species. However, mutations could freely occur in regions not contributing significantly to biological activity.

The existence of homologous regions in proteins could also be due to a convergent evolutionary process whereby the common structural features now present were independently conceived. Only through the existence of very extensive homologies could a convergent genetic pathway be rejected.

An alternative approach to the study of protein homologies is to consider different proteins from a single species. The "serine" esterases and peptidases, distinguished by their ability to react with the nerve gas diisopropylphosphorofluoridate (DFP) at a unique serine residue in the active site, lend themselves to such a study. Through the use of labelled DF^{32}P and mild acid or proteolytic hydrolysis, active centre peptides were isolated and a common sequence was established as GLY-ASP-_PSER-GLY in trypsin (130), CHT-A₄ (30,31,134), CHT-B (14), elastase (135) and thrombin (136).

Subtilisin is an excellent example of convergent evolution since it is capable of being inhibited by DFP but, unlike the other "serine" enzymes, it does not possess disulfide bridges. The sequence around the active serine has been established as THR-SER-_PMET-ALA (137). Interestingly, subtilisin is capable of being acylated by a chymotryptic reagent, indole acryloylimidazole (138).

The sequence surrounding the active serine in liver aldehyde esterase (139) and pseudocholinesterase (140) is similar to

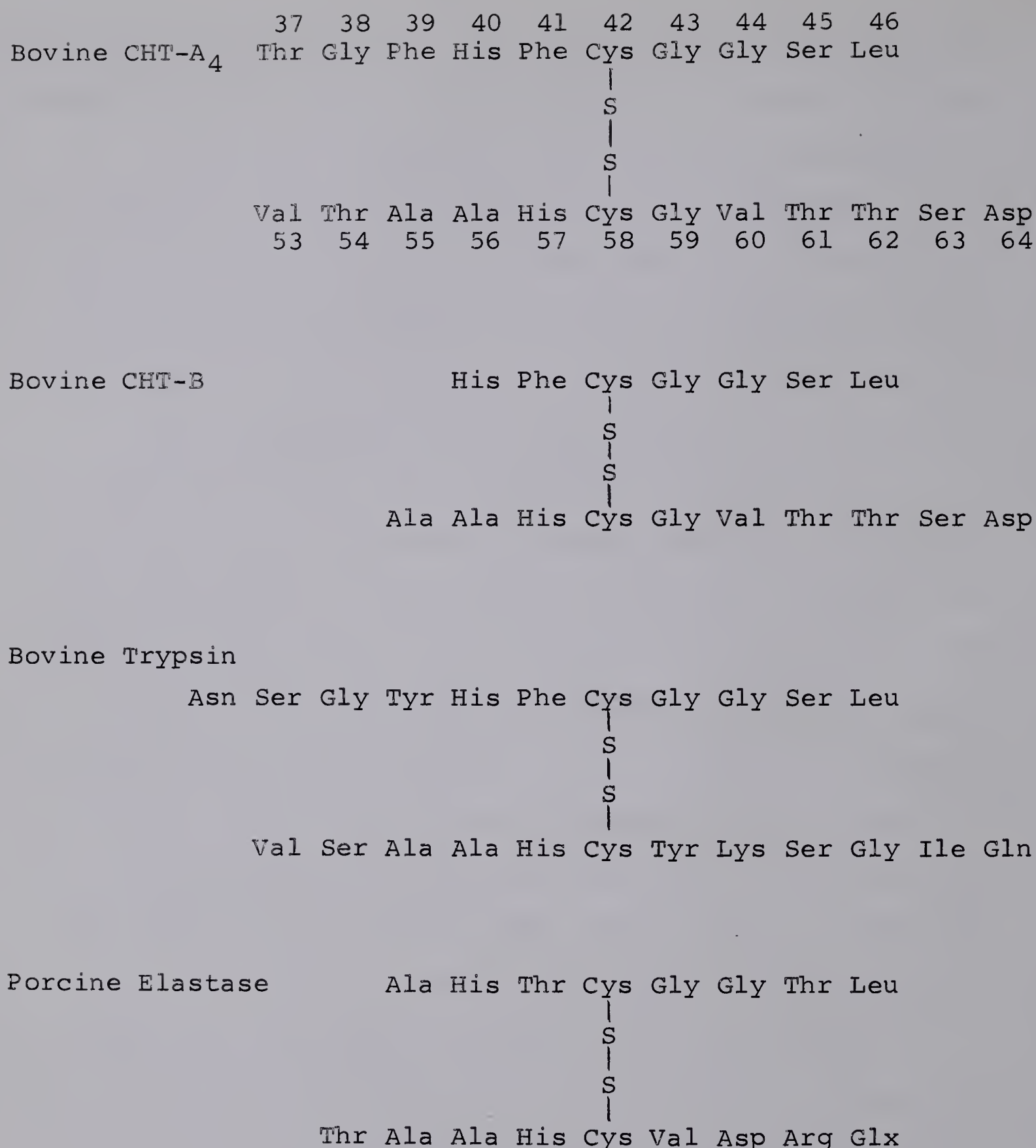


Figure 1. The amino acid sequences of di-histidine cystine peptides of bovine CHT-A₄, CHT-B, trypsin and porcine elastase.

(Smillie and Hartley (16))

other "serine" enzymes in that the aspartic acid residue is replaced by glutamic acid in an otherwise identical sequence.

Additional similarities of structure, particularly between trypsinogen and chymotrypsinogen A, did not come to light until a few years later during studies by Hartley et al. (11,12,131), Kiel et al. (133), and Walsh et al. (149,150). Application of the "diagonal" technique of Brown and Hartley (132) to the "serine" enzymes—CHT-A₄, CHT-B, elastase and trypsin—has led to the isolation of cystinyl peptides containing numerous homologies (12,16). Comparison of the di-histidine cystine peptides is presented in Figure 1. It is noteworthy that, in the enzymes studied, two histidine residues (histidine 40 and 57) are found in identical spacial relationships to each other. Moreover, it has been demonstrated that the specific alkylation of histidine-57 in CHT-A₄, by the bifunctional reagents L-TPCK (104) and PMCK (109), and in trypsin, by L-1-chloro-3-tosylamido-7-amino-2-heptanone (L-TLCK) (143), was concomitant with the loss of biological activity.

The extensive homologies which surround half-cystine-42 and precede half-cystine-58, end abruptly at residue-59. This appears to be quite significant since Hartley (11) has shown that half-cystine residues 42 and 58 in CHT-A₄ form what has been referred to as a "histidine-loop". A similar structure has been shown to exist in trypsin by Walsh et al. (149,150), thus suggesting that a "histidine-loop", maintaining two histidine residues in a specific orientation to each other, may be a common structural feature in the "serine" enzymes as has

Chymotrypsin A₄

39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	
Phe	His	Phe	Cys	Gly	Gly	Ser	Leu	Ile	Asn	Glu	Asn	Trp	Val	Val	Thr	Ala	Ala	His	Cys	

			S																	S

Trypsin

Tyr	His	Phe	Cys	Gly	Gly	Ser	Leu	Ile	Asn	Ser	Gln	Trp	Val	Val	Ser	Ala	Ala	His	Cys	

			S																	S

Figure 2. The amino acid sequence of the "histidine-loop" of CHT-A₄ and trypsin (Hartley (11), Walsh and Neurath (150)).

been shown to be the case for the GLY-ASP-SER-GLY sequence. The amino acid sequences of the "histidine-loop" in CHT-A₄ and in trypsin are compared in Figure 2.

Bender, Killheffer and Kedzy (151,152) have been impressed with the structural homology within the "histidine-loops" of CHT-A₄ and trypsin and suggest that the hydrophobic residues between half-cystines-42 and -58 might contribute to a common binding site in chymotrypsin and trypsin. Such a common hydrophobic binding site in these enzymes is supported by kinetic evidence. Thus Inagami and Sturtevant (157) demonstrated that trypsin catalyses the cleavage of certain aromatic substrates and that this activity is an intrinsic property of trypsin itself. Further, the recent finding of Inagami and Murachi (158), of Inagami (159), and of Mares-Guia and Shaw (160) indicate that the specificity site of trypsin is composed of both an anionic site and a hydrophobic slit or crevice to which the carbon side chain of substrates or inhibitors is bound. The rates of deacylation of a number of non-ionic acyl-chymotrypsin A₄ and trypsin compounds have been demonstrated to be essentially identical over a 10⁵-fold range of rate constants. This kinetic identity presumably reflects a similar interaction of the various acyl-groups with very similar specificity sites of both enzymes. The suggestion by Bender et al. (152) that this common binding site is provided by the homologous "histidine-loop" structure in both enzymes finds support from the interaction of the cyclodextrans (cyclic polysaccharides) with aromatic compounds including the chymotryptic substrate

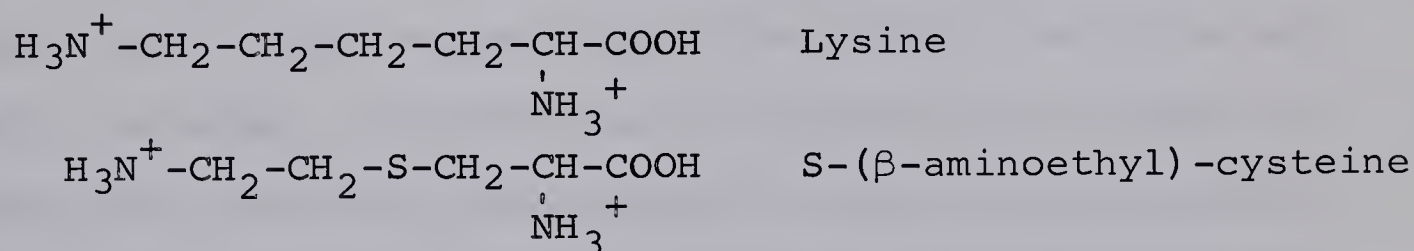
N-acetyl-L-tyrosine ethyl ester (ATEE) (144). It is postulated that the predominately hydrophobic cavity of the "histidine-loop" in CHT-A₄ would be of the proper size to interact either on the surface of the loop or within the cavity, with the aromatic groups of chymotrypsin substrates. In trypsin, of course, some additional feature of the site must explain the ionic interaction between substrate and enzyme. Since such a negatively charged grouping is absent in the "histidine-loop" of trypsin, some other region of the structure must also be invoked in this case.

An alternative view is that the "histidine-loop" of trypsin and chymotrypsin functions not as a major binding site for the substrate but for the maintenance of the exact stereochemical geometry of the catalytic site. As alternative candidates for the hydrophobic binding sites of chymotrypsin and trypsin, the areas of sequence between residues 95 and 114, between 162 and 170 and between 181 and 194 have been suggested by Smillie and Hartley (189). These homologies of sequence are accompanied by an acidic residue in trypsin which is absent in chymotrypsin.

Because of the possible significance of the "histidine-loop" of the proteolytic enzymes, either in the capacity of substrate binding sites or for the maintenance of the tertiary structures of their catalytic sites, it was considered of importance to further elucidate the sequence of this structure in CHT-B. Such information would perhaps shed further light on the importance of this structure for the enzymic activity of the pancreatic proteases.

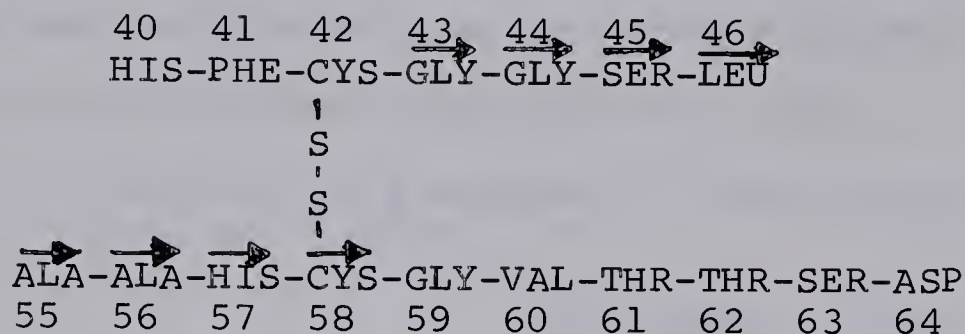
The "histidine-loops" in CHT-A₄ and trypsin were delineated by conventional techniques of overlap peptides while the entire enzyme or the isolated B chain was under study. Such an approach is laborious and a multitude of peptides must be examined before those peptides comprising the "histidine-loop" could be ultimately sequenced. A more direct approach was desired for studies on CHT-B.

Lindley (161) has described a method by which trypsin could be directed towards the catalysis of peptide bonds originally involving cysteine or cystine residues. Aminoethylation of the sulfhydryl group of cysteine (free or reduced cystine) with bromoethylamine produced a new amino acid, S-(β-aminoethyl)-cysteine (AE-cysteine), which was almost identical to lysine except that a methylene group at position 4 was replaced by a sulfur atom.



Since the sulfur atom occupies approximately the same volume as a -CH₂- group and since the bond angles of these groups are similar (sulfur = 100°; tetrahedral carbon = 109.5°), the modified cysteine residue could mimic lysine and thus pose as a tryptic substrate. Application of this technique has been reported by Hofmann (162) and Jones (163) working with trypsino-gen and haemoglobin, respectively. During the time attention was being directed to the "histidine-loop" of CHTG-B, Smillie

and Hartley (16,17) had determined the amino acid sequence surrounding the disulfide bridge (42-58) which brings the two histidine residues into juxtaposition.



A knowledge of the N-terminal (residues 43-46) and C-terminal (residues 55-58) sequences of the "histidine-loop" (residues 43-58) was thus available. (See arrows →)

A method by which the "histidine-loop" could be isolated from CHTG-B as a single peptide now presented itself. The zymogen could be reduced using mercaptoethanol in urea and aminoethylated with bromoethylamine ($\text{Br} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{NH}_3^+ \text{Br}^-$). S-(β -aminoethyl)-chymotrypsinogen B (AE-CHTG-B) would be subsequently obtained. Digestion with trypsin would cleave all peptide bonds involving lysine, arginine and S-(β -aminoethyl)-cysteine (AE-cysteine). The "histidine-loop" peptide could be isolated intact, provided it was void of lysine and arginine. This was true for CHTG-A and was likely the case in CHTG-B. The presence of ten additional cleavage points in the zymogen would favour the absence of large, insoluble, tryptic "cores", thus aiding in the purification of the peptide sought. Identification of the "histidine-loop" peptide in the tryptic digest was facilitated by the presence of two "handles"—histidine and tryptophan—both of which could be

readily detected by color reactions (Appendix B).

Theoretically, the isolation of the "histidine-loop" peptide from CHTG-B appeared to be straightforward and few obstacles were envisaged. As the work progressed, it became obvious to both student and supervisor that:

"... the best-laid schemes o' mice and men
Gang oft a-gley,..."

To A Mouse - Robert Burns.

2. Reduction and Aminoethylation of Chymotrypsinogen-B

a. Methods

(i) Bromoethylamine.Hydrobromide Procedure

Chymotrypsinogen-B prepared by the procedure of Enenkel et al. (141) was used as the starting material for the isolation of the "histidine-loop". Reduction of CHTG-B (300 mgs) was performed as outlined by Canfield and Anfinsen (164). The zymogen (1.2×10^{-5} moles) was added to 30 mls of 10 M urea, pH 3.0, previously flushed with nitrogen, and the solution was allowed to remain at room temperature for thirty minutes. Following the addition of 500 μ l of mercaptoethanol (Eastman Organic Chemicals), the pH was adjusted to 8.0 with 6N NH_4OH , sealed under nitrogen and the solution placed at 37° for four hours. The material was then transferred to a centrifuge tube, previously flushed with nitrogen, stirred and precipitated with 225 mls of ethanol-hydrochloric acid (98/2; v/v) at -20° for sixteen hours. The gelatinous protein pellet obtained after one hour of centrifugation at 9000 rpm was quickly transferred to a 50 ml beaker and dissolved in 30 mls of 8 M urea, pH 3.0,

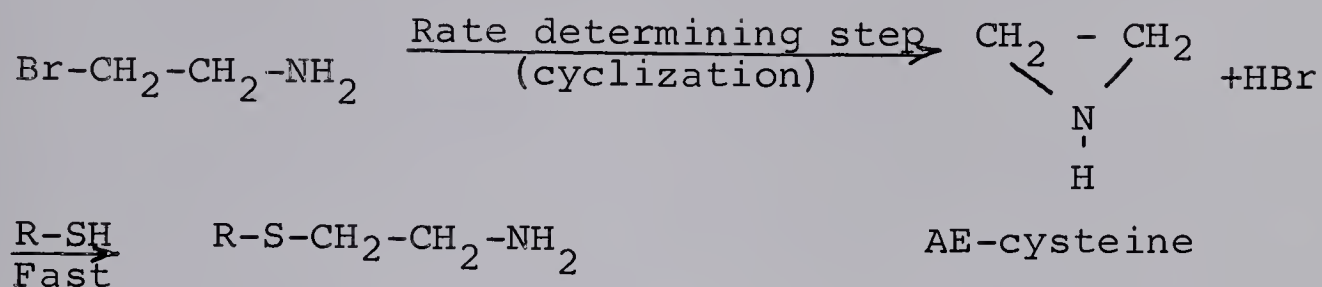
under an atmosphere of nitrogen.

Aminoethylation of reduced CHTG-B was initiated by introducing 1.23 gms of bromoethylamine.HBR (maximum 50/1 per sulfhydryl group) (Eastman Organic Chemicals), followed by careful adjustment of the pH to 8.0. The reaction was arbitrarily carried out for 4 hours at 25⁰, 1 hour at 37⁰, and completed with an overnight incubation at 10⁰. During the lengthy alkylation period, the pH was periodically adjusted to 8.0 with 6N NH₄OH. Adjustment of the solution to pH 3.0 followed by 24 hours dialysis against 4 x 3 litres of 10⁻³M HCl at 5⁰ and lyophilization, produced 264 mgms of S-(β-aminoethyl)-CHTG-B (AE-CHTG-B) in an 88% yield. Amino acid analyses indicated a recovery of 8.9 out of a maximum of 10 residues (Table I). The loss of two methionine residues was confirmed by the presence of homoserinelactone—a degradation product of methionine sulfonium salts (Fig. 37CH4).

Attempts to isolate the "histidine-loop" peptide from AE-CHTG-B were largely unsuccessful, yielding minute quantities from which only a crude representation of the amino acid composition was obtained. The difficulties were ultimately traced to the presence of chymotrypsin in the trypsin digest and instability of the peptide during ionophoresis. A discussion of the purification procedures will be presented in section 4.

A review of the mechanism by which bromoethylamine led to the aminoethylation of a sulfhydryl group indicated the

existence of a cyclic intermediate, ethylenimine, as the reactive species (165,166).



Raftery and Cole (198) have compared the reaction rates of ethylenimine and bromoethylamine with reduced glutathione and have shown the former to be 60-fold more reactive than the latter. They further indicated, by amino acid analysis, that exposure of the reduced B chain of insulin to a 100 molar excess of ethylenimine led only to the alkylation of cysteine. Since methionine is absent in insulin, the possibility of sulfonium salt formation was not encountered.

Through the use of bromoethylamine.HBr as the amino-ethylating reagent, Br^- was introduced into the medium to a maximum concentration of 0.4 M. Traces of peroxides or heavy metals could initiate formation of free bromine which in turn could lead to deleterious side reactions, particularly at the tryptophan residues (199). Ethylenimine was used as the amino-ethylating reagent in all subsequent work since the absence of Br^- and the rapid reaction with sulfhydryl groups indicated it was the preferred reagent.

(ii) Ethylenimine Procedure

The preparation of S-(β -aminoethyl)-CHTG-B, as outlined below, was adopted for routine use in the laboratory.

TABLE I

The Amino Acid Composition of
S-(β -aminoethyl)-Chymotrypsinogen B

Amino Acid	Bromoethylamine Preparation	Ethylenimine Preparation	CHTG-B ^d	CHTG-B ^e
Lysine	11.3	10.9	10.6	10.9
Histidine	2.0	2.1	2.0	2.1
Arginine	5.8	3.9	5.1	5.0
AE-cysteine ^c	8.9	8.3	-	-
Aspartic Acid	19.2	20.8	19.5	19.8
Threonine	20.9 ^b	23.6 ^b	20.3	22.2
Serine	19.7 ^b	21.2 ^b	20.6	21.7
Glutamic Acid	17.6	18.9	18.5	19.4
Proline	12.5	11.2	12.7	15.1
Glycine	22.0	22.8	22.4	23.3
Alanine	22.0 ^a	22.0 ^a	22.0	23.0
Half-cystine	-	-	9.5	9.6
Valine	22.5	22.2	23.5	23.9
Methionine	2.0	3.4	3.8	3.8
Isoleucine	7.7	7.7	8.5	8.3
Leucine	17.7	18.2	18.6	18.7
Tyrosine	2.9	3.3	3.2	3.3
Phenylalanine	6.5	7.8	6.8	6.8
Homoserine Lactone	Trace	Trace	-	-

a. Arbitrarily taken as 22.0 residues

b. Not corrected for hydrolytic destruction

c. Integration constant 46.6

d. Data of Smillie et al. (20)

e. Data of Guy et al. (195)

Reduced CHTG-B (300 mg prepared as discussed in section (i)) was dissolved in 30 mls of 8 M urea, pH 3.0, at 10° under nitrogen. A calculated amount of glacial acetic acid (150 μ l) was added to the solution prior to the addition of 620 μ l of ethylenimine (0.4 M, Matheson, Coleman and Bell Co.) in order to achieve a final pH near 8.0. The use of an acetate-ethylenimine buffer system near pH 8.0 ensures the rapid aminoethylation of sulfhydryl groups (ratio of ethylenimine/sulfhydryl of 100/1) while preventing the alkylation of α -amino groups which readily occurs above pH 9.0. After allowing the aminoethylation to proceed for one hour at 25°, the solution was brought to pH 3.0 with 6N HCl and dialysed for 24 hours against 4 x 3 litres of 10⁻³ M HCl at 5°. Lyophilization yielded 250 mg of AE-CHTG-B in 84% yield. Amino acid analysis indicated the recovery of 8.3 residues of AE-cysteine. The loss of methionine was substantiated by the presence of homoserine lactone. A comparison of AE-CHTG-B prepared with bromoethylamine.HBr and ethylenimine is presented in Table I and shows 89% and 83% recovery of AE-cysteine, respectively. The data indicates the aminoethylated CHTG-B preparations are similar.

Clearly, the recovery of AE-cysteine from AE-CHTG-B upon acid hydrolysis could reflect a true measure of the extent of aminoethylation provided, however, that AE-cysteine was stable during the hydrolysis. Knowledge of the integration color constant, used to calculate the number of micromoles of AE-cysteine, was of vital importance in these studies.

To eliminate ambiguities, AE-cysteine was synthesized from L-cysteine and ethylenimine by the method of Cavallini et al. (200) as shown in Appendix D. The integration constant was found to be 46.6 compared with 51.2 for lysine. It was found to be stable in 6N acid at 110⁰ for a minimum of 72 hours—its stability being unaltered by the presence of a protein hydrolysate. Recovery of AE-cysteine from AE-CHTG-B was 89% and 83% from preparations (i) and (ii), respectively. The latter figure is not in agreement with the data of Hofmann (162), who reported 95% recovery of AE-cysteine from aminoethylated trypsinogen. Several preparations of S- β -aminoethyl-CHTG-B, using ethylenimine as the aminoethylation reagent, consistently yielded approximately 85% recovery of AE-cysteine. The reason for this discrepancy is not known.

3. Tryptic Digestion of S-(β -aminoethyl)-chymotrypsinogen-B

a. Procedure

Contaminating chymotrypsin in the trypsin preparation (Worthington, twice crystallized TR836-38) was inactivated with L-TPCK according to the method of Kostka and Carpenter (201). The tryptic digestion of AE-CHTG-B was performed at pH 8.0, 25⁰, using a Radiometer Titrigraph type SBR2C coupled with a Radiometer TTT-1a titrator. The aminoethylated enzyme (140 mg) was dissolved in 30 mls of 0.01 M HCl by stirring for one hour with the aid of a magmix. A precipitate appeared near pH 6 as the pH was slowly adjusted to pH 8.0 by the addition of 0.3 N NaOH. This likely corresponds to the

isoelectric point of AE-CHTG-B. Trypsin (3 mgs; L-TPCK treated) was added as a 1% solution in water to the suspension and a pH of 8.0 was maintained automatically by the addition of 0.3N NaOH. The precipitate initially present was solubilized within an hour. The progress curve of the tryptic digestion showed an initial rapid uptake of base followed by a gradual tapering off in the rate of hydrolysis. After approximately 85% of the tryptic susceptible bonds had been cleaved (7 hours), the digestion was terminated by adjusting the pH to 3.0. More extended digestion periods would have only favoured chymotryptic cleavages.

b. Results and Discussion

Early experiments on the tryptic digest (not treated with L-TPCK) of AE-CHTG-B revealed, following pH 6.5 ionophoresis, two major Pauly bands (histidine peptides) migrating rapidly towards the cathode and a weak Pauly band in the neutral region. Since only two histidine residues existed in the enzyme, it was optimistically assumed that one of the basic Pauly bands must represent the "histidine-loop" peptide. Purification of the bands was accomplished with few difficulties and amino acid analyses were performed. Instead of the intact "histidine-loop" being present, only a fragment encompassing histidine-57 was present. Using the studies of Smillie et al. (16,17) as reference, the Pauly positive peptides could be identified as HIS(40)-PHE-AE.CYS and ALA-ALA-HIS(57)-AE.CYS. The latter peptide was the most basic and actually existed as two discrete bands which possessed similar compositions

differing only in the AE-cysteine content. It is noteworthy that the above peptide was later isolated from peptic and chymotryptic digests of the purified "histidine-loop" peptide and was always found as two or three bands.

As was indicated above, a weak Pauly band was present in the neutral region and attempts to determine the composition of this peptide are presented in section 4.

The cause of the deleterious cleavages was suspected to be chymotryptic contamination of the trypsin. This was verified by Dr. Arpád Fürka (working in this laboratory) during sequence studies on peptides derived from a tryptic digest of AE-CHTG-B. He isolated an acidic, tryptophan containing, peptide which possessed an amino acid composition not inconsistent with that of the "histidine-loop" minus the C-terminal fragment ALA-ALA-HIS-AE.CYS.

Comparison of the pH 6.5 ionophoresis patterns of the tryptic digest using L-TPCK treated and untreated trypsin, revealed marked differences. Two new Pauly positive peptides, present in the neutral region and at the origin, replaced the basic peptides previously isolated. A trace of the ALA-ALA-HIS-AE.CYS peptide persisted, indicating the presence of a particularly CHT-A₄ sensitive bond in the "histidine-loop". The mobilities of the new histidine peptides implied an increase in their molecular weight. This, coupled with the coincidence of a Pauly positive and Ehrlich positive (tryptophan containing) band in the neutral region was assurance that the "histidine-loop" was now intact.

4. Isolation and Purification of the "Histidine-Loop"

a. Preliminary Experiments

Prior to the inactivation of chymotrypsin in the trypsin preparations, the "histidine-loop" peptide was obtainable from the neutral band only in minute quantities. Utilizing a combination of pH 6.5 ionophoresis, gel filtration of the pH 6.5 neutral band on Sephadex G-25, and pH 1.8 ionophoresis of the Pauly positive peaks, obtained following gel-filtration, an amino acid composition of the "histidine-loop" peptide was obtained which indicated minor differences from CHT-A₄. The data is in accordance with subsequent analyses (see Table II). The low recoveries of histidine and AE-cysteine are thought to reflect degradation during the extensive ionophoresis purification. Since sequential studies on the "histidine-loop" could not possibly be conducted on the material so far isolated, the purification procedures were reviewed. The treatment of trypsin with L-TPCK was undoubtedly the most significant step in the purification procedure for the "histidine-loop" peptide.

(i) Ionophoretic Purification

Increased yields of the "histidine-loop" peptide in the neutral band indicated the possibility of using ionophoresis as the sole means of purification.

The tryptic digest (L-TPCK treated trypsin) of AE-CHTG-B (40 mg) (ethylenimine preparation) was spotted at a concentration of 1.0 mg/cm, 21 cm from the anode on a full sheet of Whatman #3 MM paper. Ionophoresis was performed at pH 6.5, 50 v/cm for 70 minutes. Side strips (1 cm) were developed with ninhydrin, Pauly and Ehrlich reagents to identify the various bands.

Three histidine bands were revealed by the Pauly reaction--a trace of a very basic peptide migrating behind lysine; the dominant "histidine-loop" peptide in the neutral region; and a dominant peptide, likely containing histidine-40, remaining at the origin. Tryptophan was found to coincide with histidine in the neutral region. Strips of the neutral region were subjected to ionophoresis at pH 1.8, 70 v/cm for 35 minutes, and developed with the appropriate color reagents. Once again the histidine and tryptophan spots coincided, while the ninhydrin map showed the presence of at least two peptides migrating with the "histidine-loop" peptide. The neutral band was re-run at pH 6.5, 3Kv for 5-1/2 hours in an attempt to remove these contaminants. The "long 6.5" ionophoresis improved the separation of the peptides, but unexpectedly led to a marked decrease in the intensities of the Pauly and Ehrlich color reactions. Peptide maps obtained through "long 6.5"-1.8 ionophoresis indicated only traces of a Pauly positive spot and its corresponding ninhydrin spot while evidence for tryptophan was absent.

An unknown side reaction, prevalent during ionophoresis at pH 6.5 and to a lesser extent at pH 1.8, likely led to an alteration in the net charge on the "histidine-loop" peptide, thereby decreasing its mobility and causing the peptide to be spread over the entire paper. That the site of this side reaction was the AE-cysteine residue was supported by studies on the peptic and chymotryptic peptides of the "histidine-loop" (sections 5b and 5c). Attempts to isolate the "histidine-loop" peptide solely by ionophoretic techniques were abandoned in

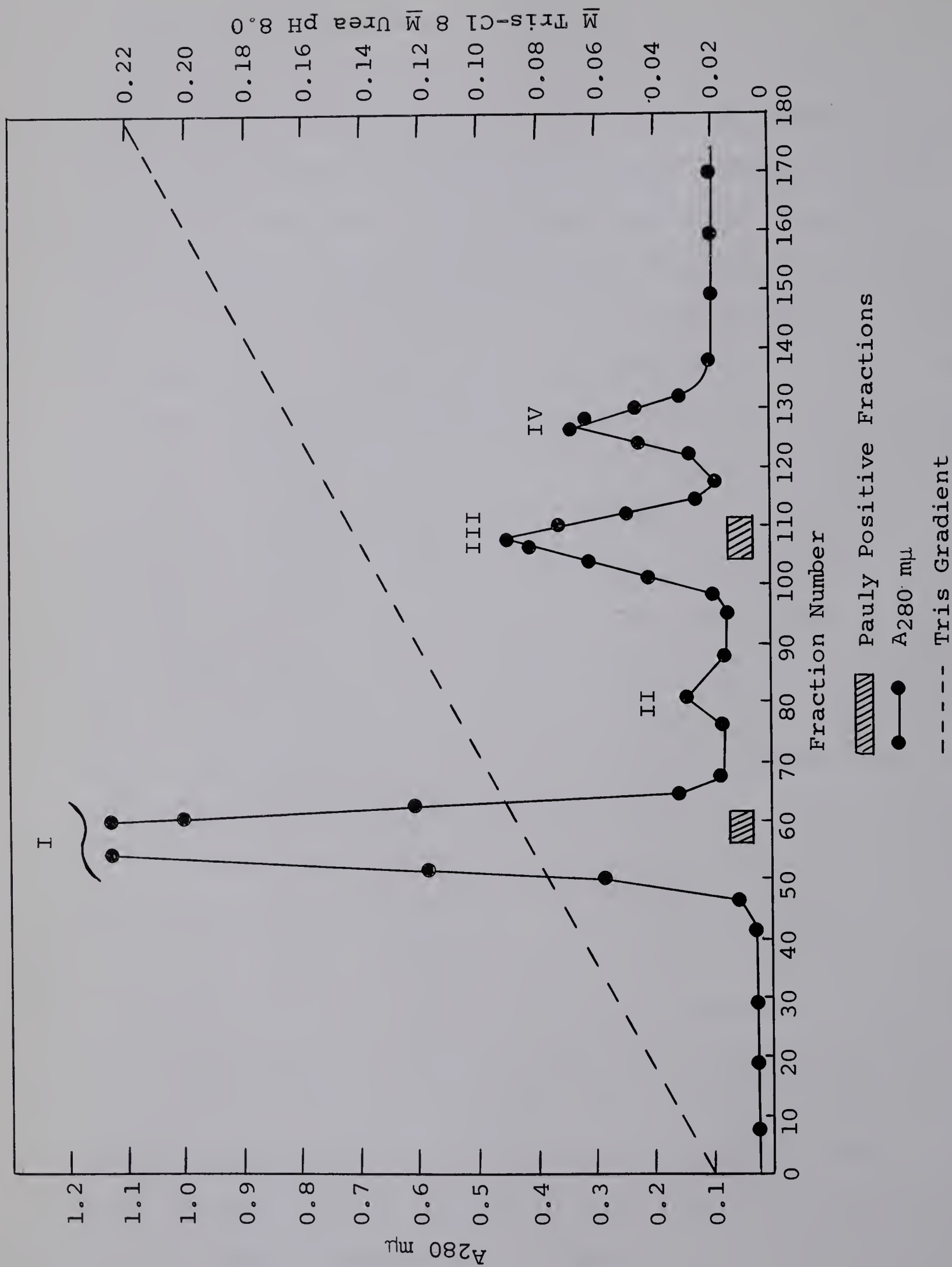


Figure 3. DEAE-cellulose chromatography of the tryptic digest of S-(β -aminoethyl)-chymotrypsinogen B.

favour of ion exchange chromatography and gel filtration.

(ii) Chromatographic Purification

Ion exchange chromatography in conjunction with gel filtration offers considerably greater peptide recovery, albeit the procedures are more time consuming and tedious than purification using ionophoretic techniques. It was decided to adopt a procedure successfully applied by Hartley (72) to the separation of tryptic "core" peptides from the B chain of CHT-A₄. DEAE-cellulose (0.9 meq per gm) was prepared in 0.02 M tris-HCl buffer, 8 M urea, pH 8.0. Prior to use, the 8 M urea was deionized by passing it through a mixed bed resin (Dowex 1-50) in order to remove cyanate (202). A tryptic digest of AE-CHTG-B (120 mg) in 10 mls of starting buffer was added to a column of DEAE-cellulose (2.6 x 67 cm) previously equilibrated with one litre of 0.02 M tris-HCl buffer, 8 M urea, pH 8.0. The digest was washed in with two 3 ml aliquots of starting buffer. Elution of the peptides was conducted with a linear gradient of 400 ml each of 0.02 M tris and 0.22 M tris both 8 M with respect to urea at a pH of 8.0. The flow rate was 5 mls per three minutes; 5.0 ml fractions being collected. All operations were conducted at room temperature. Based on optical density units, all material placed onto the column was recovered. The chromatogram obtained is presented in Figure 3. The existence of histidine peptides was determined by spotting aliquots from each fraction onto Whatman #3 MM paper and developing with the Pauly reagent. Peaks I and III were both found to be Pauly positive.

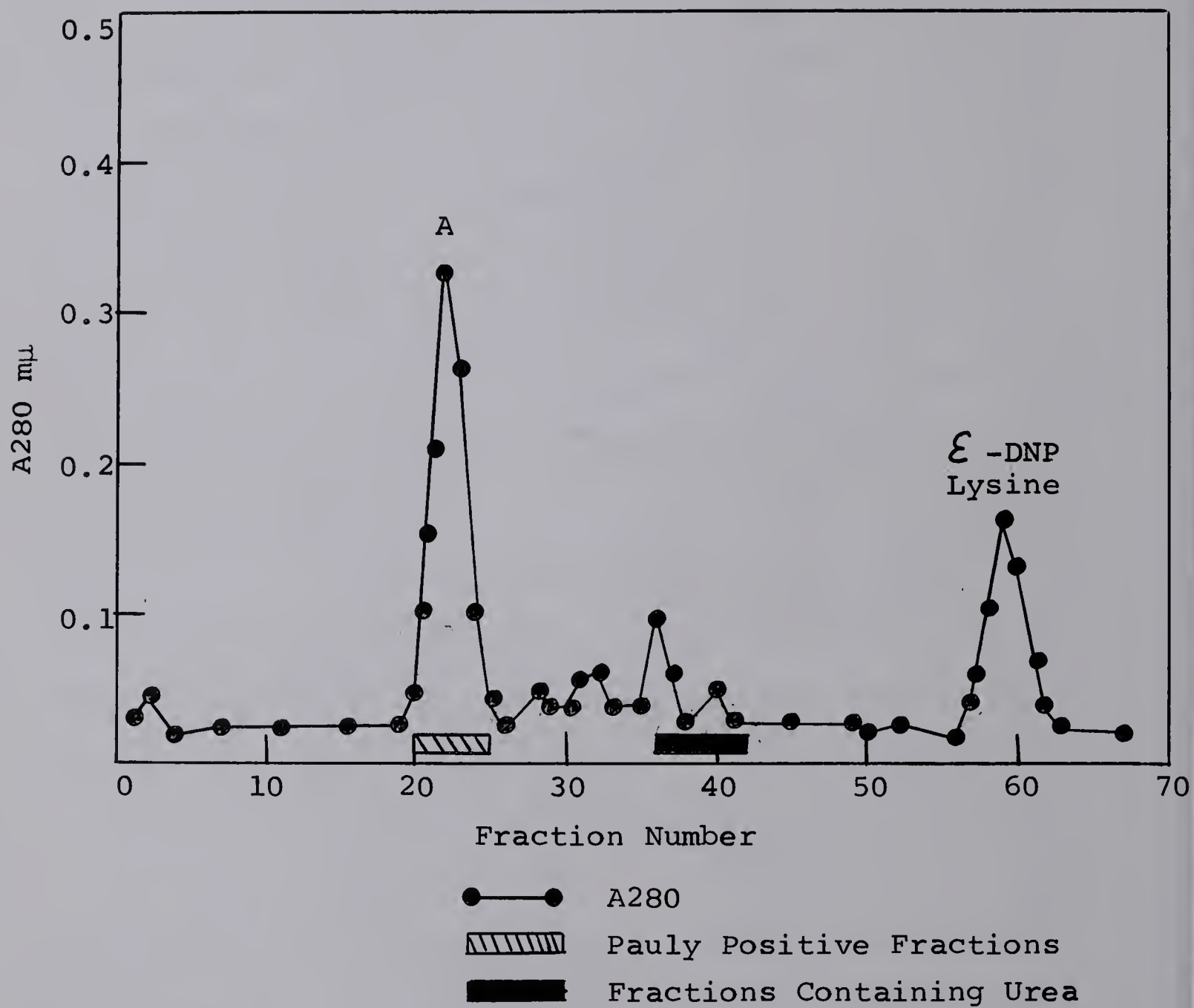


Figure 4. The removal of urea from peak III, (Fig. 3) by filtration on sephadex - G25. Flow rate 0.4 ml/min.

Since peak III appeared to be the least contaminated with ultraviolet absorbing material, it was subjected to gel filtration on Sephadex G-25 (1.1 x 125 cm) using 0.2N acetic acid (Figure 4). ϵ -DNP-lysine was used as a visual marker. Amino acid analysis of the lyophilized material under peak A (Pauly positive) was consistent with the presence of two peptides—the sixteen residue histidine loop, and a segment comprising fifteen residues of the C-terminal end of the protein. An attempt to remove the contaminating C-terminal peptide through application of Dowex-50x2 chromatography as outlined by Schroeder et al. (203) was unsuccessful. The reason for the failure was undoubtedly the instability of the "histidine-loop" peptide in the presence of pyridine-acetate buffers for extended periods of time at 38⁰. These conditions are equal to, if not more severe, than conditions during the "long 6.5" ionophoresis.

While these investigations were in progress, Dr. Arpád Fürka isolated a peptide from the C-terminal region of AE-CHTG-B. Three important facts were gleaned from his studies: (1) the peptide was acidic; (2) a tryptophan residue was present in the peptide; and (3) the amino acid composition was in agreement with that of the peptide contaminating the "histidine-loop" after gel filtration. These observations clearly explain why this peptide could not be separated from the "histidine-loop" peptide on Sephadex G-25; both peptides are approximately the same size and both contain a tryptophan residue. The fact that the C-terminal peptide was acidic

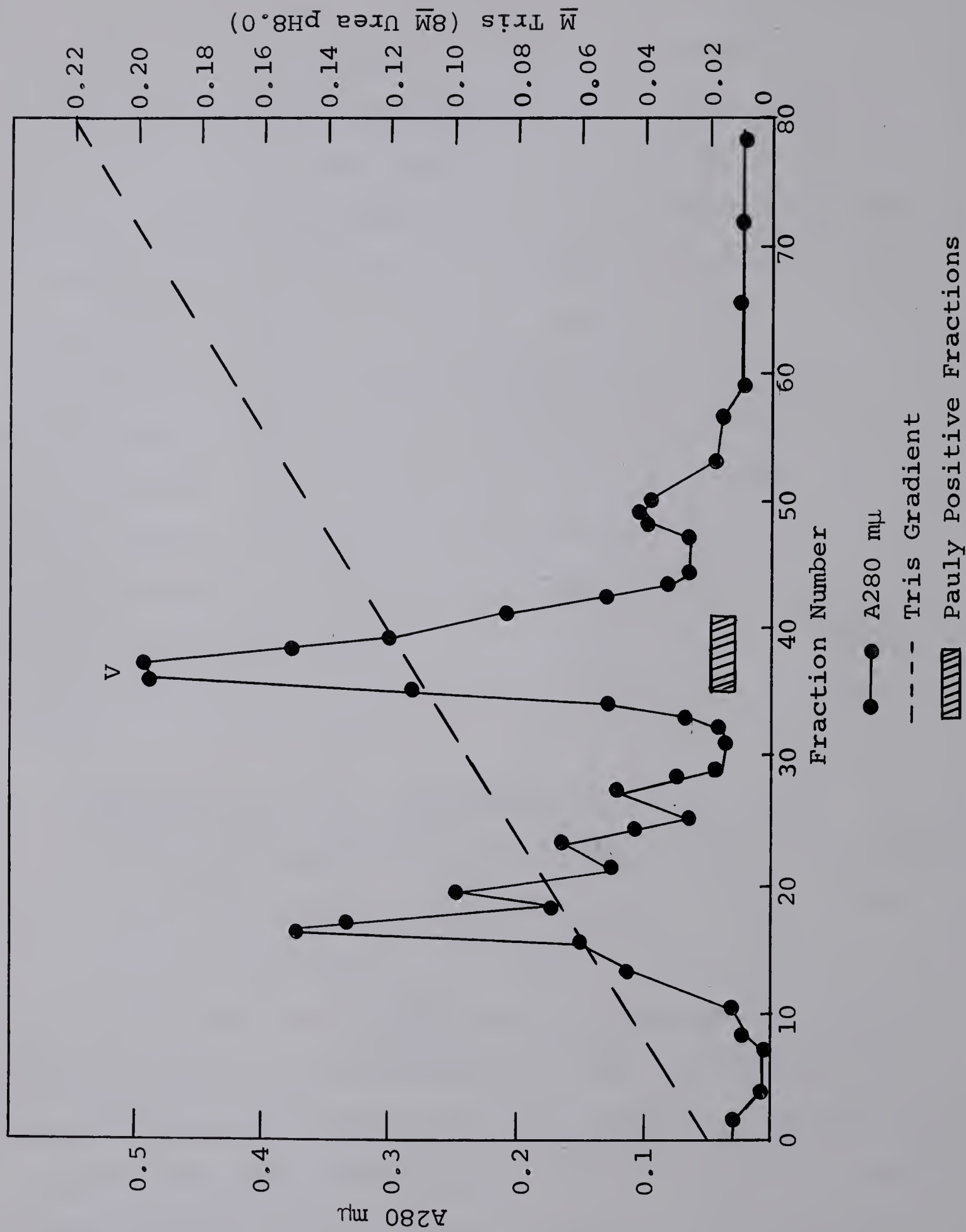


Figure 5. DEAE-cellulose chromatography of the neutral band from pH 6.5 ionophoresis of the tryptic digest of S-(β -aminoethyl)-chymotrypsinogen B.

coupled with the known properties of the "histidine-loop" during ionophoresis and DEAE-chromatography suggested a method by which the "histidine-loop" purification could be attained.

The acidic C-terminal peptide should be cleanly removed from the neutral "histidine-loop" peptide by subjecting the tryptic digest to a brief pH 6.5 ionophoresis. Elution of the peptides from the neutral region followed by chromatography on DEAE-cellulose, as outlined earlier for the intact tryptic digest, should yield the "histidine-loop" free of contaminating peptides. Removal of urea by gel filtration would complete the purification.

b. Composite Purification Procedure

The tryptic digest of AE-CHTG-B (160 mg) was spotted (1 mg/cm) in the centre of four sheets of Whatman #3 MM paper and subjected to ionophoresis at pH 6.5, 50 v/cm for one hour. The Pauly positive, Ehrlich positive, neutral band containing the "histidine-loop" peptide (4.0-5.5 cm from origin) was eluted with deionized water and lyophilized to yield 18 mgs of material. The histidine recovery at this stage was estimated at 22%.

The neutral peptides (15 mgs in 2 ml starting buffer) were chromatographed on DEAE-cellulose (0.9 x 81 cm) (0.9 meq/gm) previously equilibrated with 200 mls of 0.02 M tris.HCl, 8 M urea, pH 8.0. A linear gradient of 100 mls each of 0.02 M and 0.22 M tris, 8 M urea, pH 8.0, gave the elution pattern as shown in Figure 5. The flow rate was 11.5 mls per hour; 2.6 ml fractions were collected. Urea was removed from 10.4 mls of

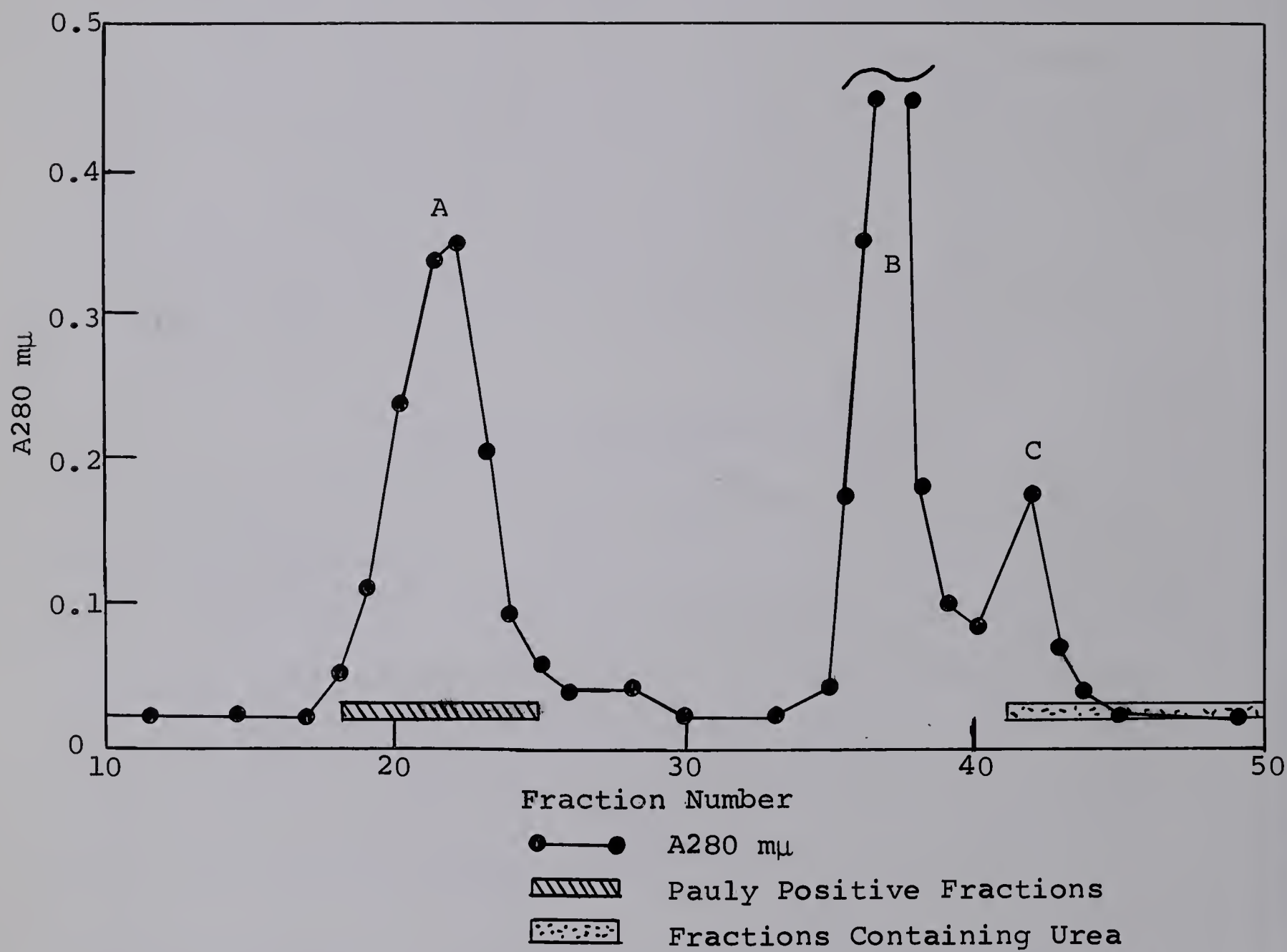


Figure 6. The removal of urea from the Pauly positive peak (Fig. 5) using Bio-Gel P-2 Flow Rate 2.8 ml/10 mins; 2.8 ml fractions.

the Pauly positive peak V on a 1.1 x 168 cm column of Bio-Gel P-2 (50-100 mesh, 1600 exclusion, Biorad) using deionized water as the eluent (Figure 6). Bio-Gel P-2 was used in preference to G-25 since the lower exclusion limit would tend to retard peptides smaller than the "histidine-loop". Skewing of Peak V, Figure 5, was indicative of impurities. These impurities are seen to be retarded on the Bio-Gel P-2 acrylamide resin and emerge as peaks B and C along with other small molecules such as urea.

The advantages of column chromatography as opposed to ionophoresis are clearly demonstrated by the recovery, based on optical density units, of over 80% of the material applied to the columns.

The "histidine-loop" peptide, Peak A, Figure 6, was lyophilized and taken up in 2.0 mls of deionized water. Amino acid analysis of a 150 μ l aliquot is presented in Table II. The "histidine-loop" of AE-CHTG-B, previously isolated by ionophoresis techniques, is also shown. Homologous structures in the "histidine-loop" region appear to exist between CHTG-A₄ and -B.

The recovery of only 1.5 residues of valine after a 22-hour hydrolysis indicates the presence of a VAL-VAL sequence since this bond is notoriously resistant to acid hydrolysis (204). The possibility of a resistant ILE-VAL or VAL-ILE bond existing in the peptide is refuted by the recovery of 1.0 residue of isoleucine.

Determination of the number of tryptophan residues in the

TABLE II

Amino Acid Analyses of the
"Histidine-loop" of AE-CHTG-B

Amino Acid	Composite a. Purification	Ionophoretic b. Purification	"Histidine-loop" c CHTG-A
Histidine	0.9	0.5	1
AE-cysteine	0.5	0.5	1
Aspartic Acid	1.0	1.3	2
Threonine	0.9	1.1 ^d	1
Serine	2.0	2.0 ^d	1
Glutamic Acid	1.0	1.1	1
Glycine	1.9	2.0	2
Alanine	2.0 ^e	2.0 ^e	2
Valine	1.5	1.9 ^f	2
Methionine	0.1	0.1	-
Isoleucine	1.0	0.9	1
Leucine	1.1	1.2	1
Tryptophan	1.2	+	1

- a. 22 hour hydrolysate
b. Average of 20 + 70 hour hydrolysate
c. Data of Hartley (11)
d. Extrapolated to zero time
e. Arbitrarily taken as 2.0 residues
f. Extrapolated to 100 hours

"histidine-loop" was calculated on the basis of amino acid analyses and the optical density reading presented in Figure 6.

The pooled fractions under Peak A had a total volume of 16.8 mls with an average extinction of 0.223. From the relationship $\epsilon = A/c \times l$, where ϵ = molar extinction coefficient, A = extinction, c = molarity, and l = cm, the molarity of tryptophan residues was calculated as 3.54×10^{-5} using $l = 1$ cm, $A = 0.223$, and $\epsilon = 6310$ at 280 m μ (205). Assuming one tryptophan and two alanine residues to be present, the quantity of the "histidine-loop" peptide was 0.60 μ moles, based on the tryptophan determination, and 0.49 μ moles based on the amino acid analysis. Since the ratio is slightly greater than unity ($0.60/0.49 = 1.2$), one may reasonably conclude that a single tryptophan residue is present. The departure of the ratio from unity is due to losses sustained during lyophilization of the "histidine-loop" following the tryptophan determination.

N-terminal analysis (section 5a(ii)) utilizing Dansyl chloride, a fluorescent reagent introduced by Gray and Hartley (206), revealed glycine in agreement with the studies of Smillie et al. (16) (Figure 1).

5. Sequence of the "Histidine-Loop" Peptide

a. Methods

The basic approach to elucidating the sequence was to degrade the peptide separately with chymotrypsin-A₄ and pepsin so as to obtain fragments which could easily be sequenced and arranged to yield over-lap peptides. The chymotryptic and peptic digestions were conducted in the volatile buffers, N-ethyl

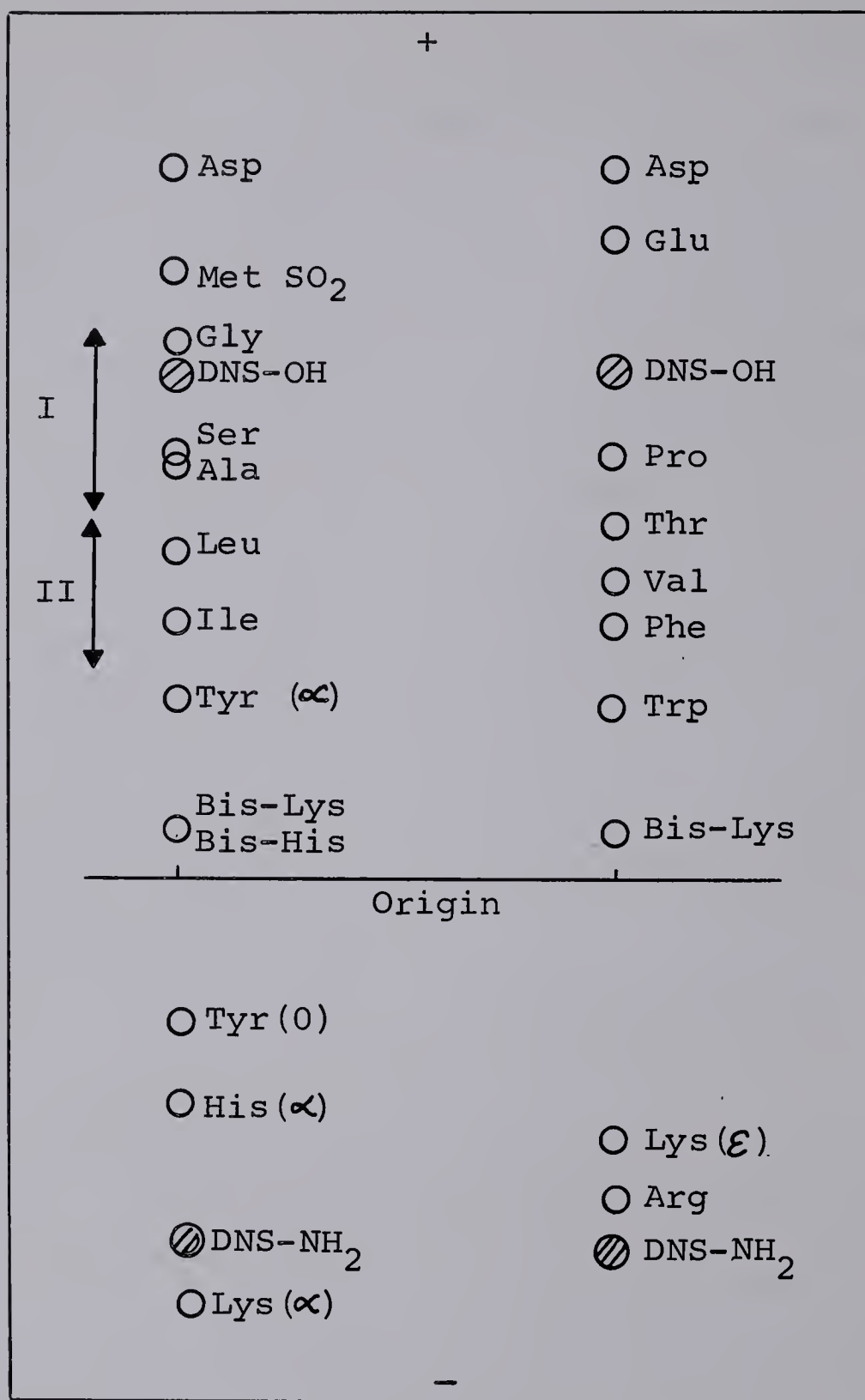
morpholine and formic acid respectively, and the digests applied directly to paper. Purification of the resulting peptides was initially carried out by ionophoresis at pH 6.5 in order to separate the peptides into basic, neutral and acidic groups. The presence of asparagine or glutamine, acidic and basic amino acids or combinations thereof, can usually be deduced from a comparison of the amino acid composition and the mobility. Cross-contaminating peptides such as those present in the neutral region, can often be resolved by subjecting the bands to ionophoresis at pH 1.8 or pH 3.5. Further resolution of bands may be obtained by descending chromatography in butanol-acetic acid-water (3:1:1, v/v/v; or 4:1:5 v/v/v), but ionophoresis at pH 1.8 is required prior to amino acid analysis (Ambler (207)).

The analysis of an N-terminal residue using Sanger's reagent, 1-fluoro-2,4-dinitrobenzene (208) has been recently superseded by an ultramicrofluorescent technique utilizing "dansyl chloride", 1-dimethylaminonaphthalene-5-sulfonyl chloride. Dansyl amino acids (DNS-AA) can be detected visually in 10^{-4} μ mole quantities by their fluorescence in ultraviolet light. This new reagent allows for a 100-fold increase in sensitivity over the 1-fluoro-2,4-dinitrobenzene technique. Thus, when used in conjunction with the Edman degradation (209-211), it is possible to carry out a sequential analysis of at least four residues using only 0.02 μ moles of peptide. A definitive treatise dealing with N-terminal analysis and sequence determination utilizing the Dansyl-Edman technique has

been written by Gray (212).

(i) Edman Degradation

Although sequential analysis is feasible with as little as 0.02 μ moles of peptide, it is advisable to use 0.05 μ moles or more of the peptide if such amounts are available. The peptide (0.05 μ moles) was evaporated to dryness in a 3.0 ml stoppered test tube and was dissolved in 200 μ l of pyridine-water (1:2, v/v). A 40 μ l aliquot (0.01 μ m) was removed for N-terminal analysis as outlined in section (ii). The volume of the residual peptide solution was brought to 300 μ l by the addition of 160 μ l of pyridine-water. The resulting solution was stored at -20° until the N-terminal amino acid had been successfully identified. A systematic degradation of the peptide was performed by introducing 300 μ l of phenylisothiocyanate (PITC) (5% in pyridine; Matheson, Coleman and Bell Co.) to the stock peptide solution. The mixture was briefly agitated, stoppered and incubated at 37° for 3 hours to achieve coupling. Deionized water (200 μ l) was added following the incubation. Pyridine and excess PITC were extracted with four 1.5 ml aliquots of benzene by first mixing thoroughly on a Junior Vortex agitator, followed by centrifugation for four minutes at three-quarter speed on an International Clinical centrifuge. The upper benzene layer was removed with a Pasteur pipette and discarded. The aqueous solution remaining was freeze-dried overnight. Early the next day, 100 μ l of trifluoroacetic acid (TFA-Baker Analyzed reagent) was added to the dried residue. Cyclization and cleavage of the PTC-peptide yielding a shorter peptide



DNS-OH = Dansyl Sulfonic Acid
 DNS-NH₂ = Dansyl Sulfonamide

Figure 7. pH 4.38 ionophoresis of dansyl-amino acids
 hatched spot indicate blue fluorescence.

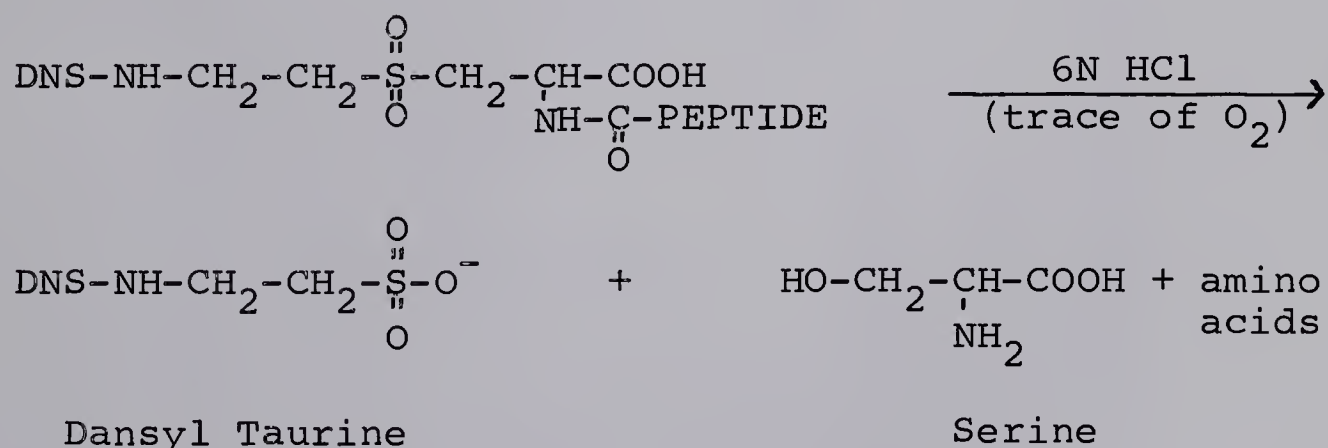
The optimum current drawn during the run has been found to be between 100 to 150 mamps. Separation of the dansyl amino acids obtained through ionophoresis is shown in Figure 7. The dansyl sulfonic acid region I, (Figure 7) may be further resolved by sewing the band onto #3 MM paper and re-running it at pH 1.8, 4 Kv for sixty minutes in the varsol-cooled ionophoresis tank (213). Under these conditions, DNS-GLY and DNS-ALA were slightly separated. DNS-SER, migrating behind DNS-ALA, was well separated, while DNS-PRO was extracted into the varsol and lost. Dansyl sulfonic acid (DNS-OH) possesses a net charge of zero at pH 1.8 and moves only slightly from the origin. Clearly, re-running of region I need only be invoked to distinguish between DNS-SER and DNS-ALA, or to remove DNS-OH, which could be masking DNS-GLY.

Confirmation of the hydrophobic dansyl amino acids migrating in region II, Figure 7, may be readily obtained by chromatography (sixteen hours) in light petroleum (100-120^o b.p.) -toluene-acetic acid-water (75:25:85:15, v/v/v/v) (216). The upper phase was used for chromatography, while the lower phase was used for pre-equilibration of the paper.

Pertinent to the sequence studies on the "histidine-loop" and other peptides from AE-CHTG-B, was the presence of N-terminal tryptophan and C-terminal AE-cysteine residues. The known lability of tryptophan to acid hydrolysis is not altered in the dansyl tryptophan derivative. Upon exposure to 6N HCl at 110^o DNS-TRP decomposes to yield dansylsulfonamide (DNS-NH₂) plus traces of DNS-OH, DNS-SER, DNS-GLY and DNS-ALA (212). Dansyl tryptophan can be readily liberated from a DNS-peptide through the use of chymotrypsin (section 5c).

During sequence studies conducted on tryptic peptides from S-(β -aminoethyl)-CHTG-B, an acidic dansyl derivative with a mobility mid-way between DNS-ASP and DNS-GLU appeared occasionally in addition to the expected N-terminal residue. A careful examination of numerous N-terminal analyses revealed that only those peptides containing AE-cysteine formed the artifact during acid hydrolysis of the DNS-peptide.

A likely explanation for its occurrence is as follows: during dansylation, the ϵ -amino group of AE-cysteine was labelled as was known to occur with the ϵ -NH₂ of lysine (212). Oxidation of the sulfur to the sulfone, via the sulfoxide intermediate, could occur during peptide purification, dansylation, and acid hydrolysis in the partially degassed HCl. Cleavage of the thioether bond as indicated below would yield dansyl taurine an acidic species with a mobility identical to the artifact (212).



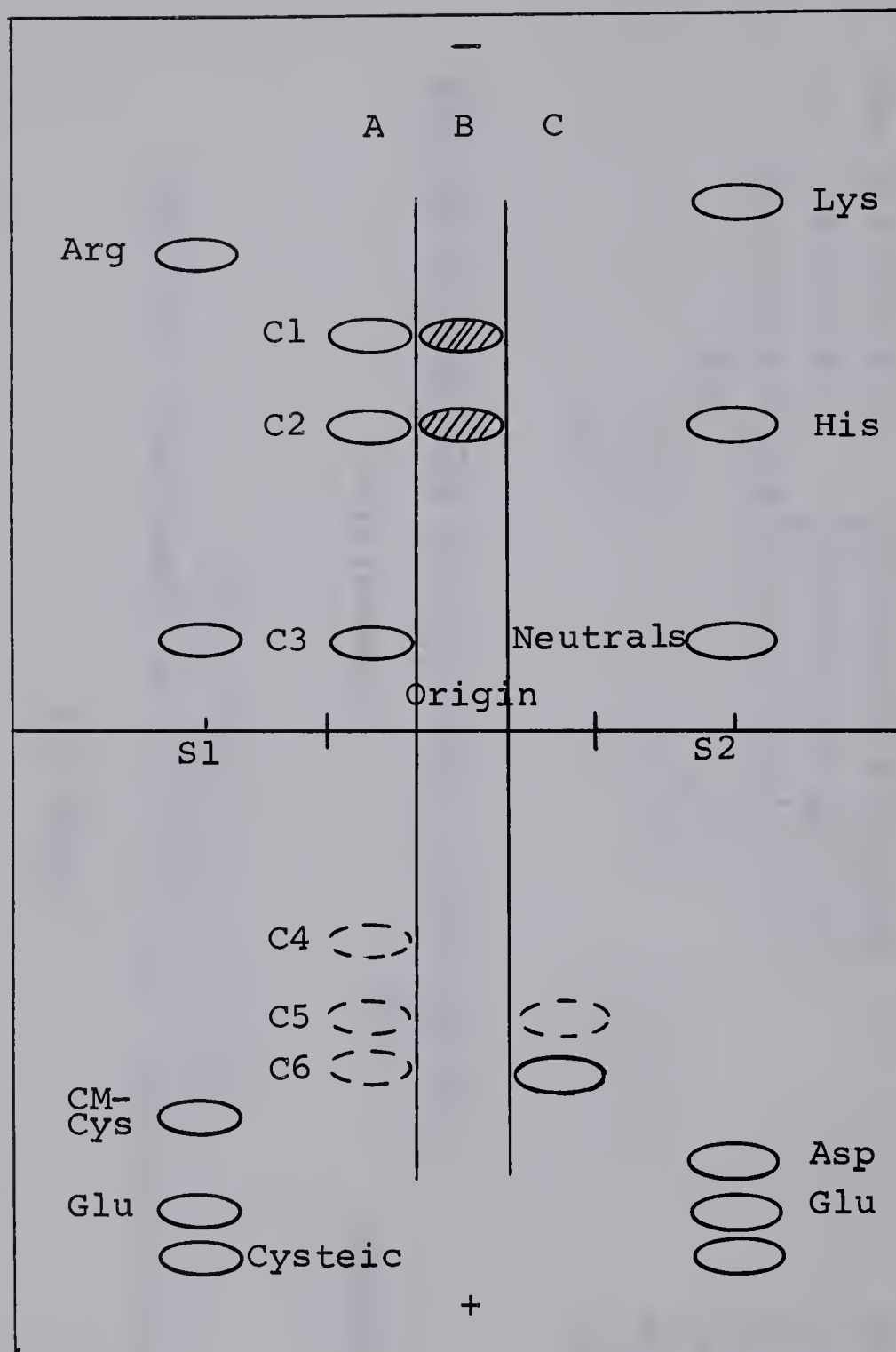
Since oxidation of synthetic AE-cysteine occurred at room temperature (Appendix D), and since multiple bands of AE-cysteine peptides have been observed (Figure 8), the proposed degradation of ϵ -DNS-S(- β -aminoethyl)-cysteine is conceivable.

TABLE III

N-Terminal and Amino Acid Analyses of Chymotryptic Peptides
from the "Histidine-loop"

Peptide	N-Terminal	Composition													
		AE		His	Cys	Asp	Thr	Ser	Glu	Gly	Ala	Val	Ile	Leu	Trp
C1	Ala		1.2	1.0							2.0 ^a				
C2	Ala		0.9	0.3		0.3	0.3				2.0 ^a				
C3	Val					1.0 ^a					1.6				
C4	Gly				1.1	0.5	2.2	1.1	1.1	1.9	2.0 ^a	0.6	0.7	0.9	
C5	Gly				1.1	1.0	1.6	1.1	2.0 ^a			1.6	1.1	1.1	+
C6	Gly				1.0	0.1	1.8	1.0	2.0 ^a			0.2	0.9	1.0	+++

a. Arbitrarily taken as reference standard



A = Cadmium-ninhydrin
 B = Pauly Reaction (histidine)
 C = Ehrlich Reaction (tryptophan)

Figure 8. pH 6.5 Ionophoresis of the chymotryptic digest of the "histidine-loop" peptide.

b. Chymotryptic Digestion of the "Histidine-Loop" Peptide

(i) Purification of Peptides

An equal volume of 0.02N N-ethylmorpholine buffer pH8.0 was added to 0.30 μ moles of the histidine loop peptide in 1.2 mls of water. Digestion was initiated by the addition of 50 μ l of a stock CHT-A₄ solution (6 mgs/2.0 ml H₂O) and incubated at 37° overnight. The molar ratio of "histidine-loop"/CHT-A₄ was 50/1.

The digest was spotted at a concentration of 0.01 μ moles/cm in parallel with standard amino acid mixtures S1 and S2 (Appendix A) in the center of a Whatman #1 paper. Ionophoresis was carried out at pH6.5 and 50 volts/cm for fifty minutes. Side strips one cm. in width were stained with Pauly, Ehrlich and ninhydrin reagents - the results are summarized in Figure 8. Rerunning the neutral band (C3) at pH1.8, 80 volts/cm for thirty minutes, revealed only one major band running slightly ahead of phenylalanine. Peptides C₁ to C₆ were eluted from their respective chromatograms with 400 μ l of water. The results of N-terminal and amino acid analysis on the peptides are presented in Table 3. Of the chymotryptic peptides isolated, only C3 (VAL, VAL, THR.) was useful in the elucidation of sequences. The complete sequences of peptides C1 and C2 and the sequences of the first four residues of peptides C4 to C6 were known previously from the work of Smillie and Hartley (16, 17). Useful overlapping peptides were obtained from the digest as shown in Figure 9. The assignment of peptide C4 to the position shown in Figure 9 was based on a questionable amino acid analysis due to poor recoveries of the peptide. The presence of this peptide in trace

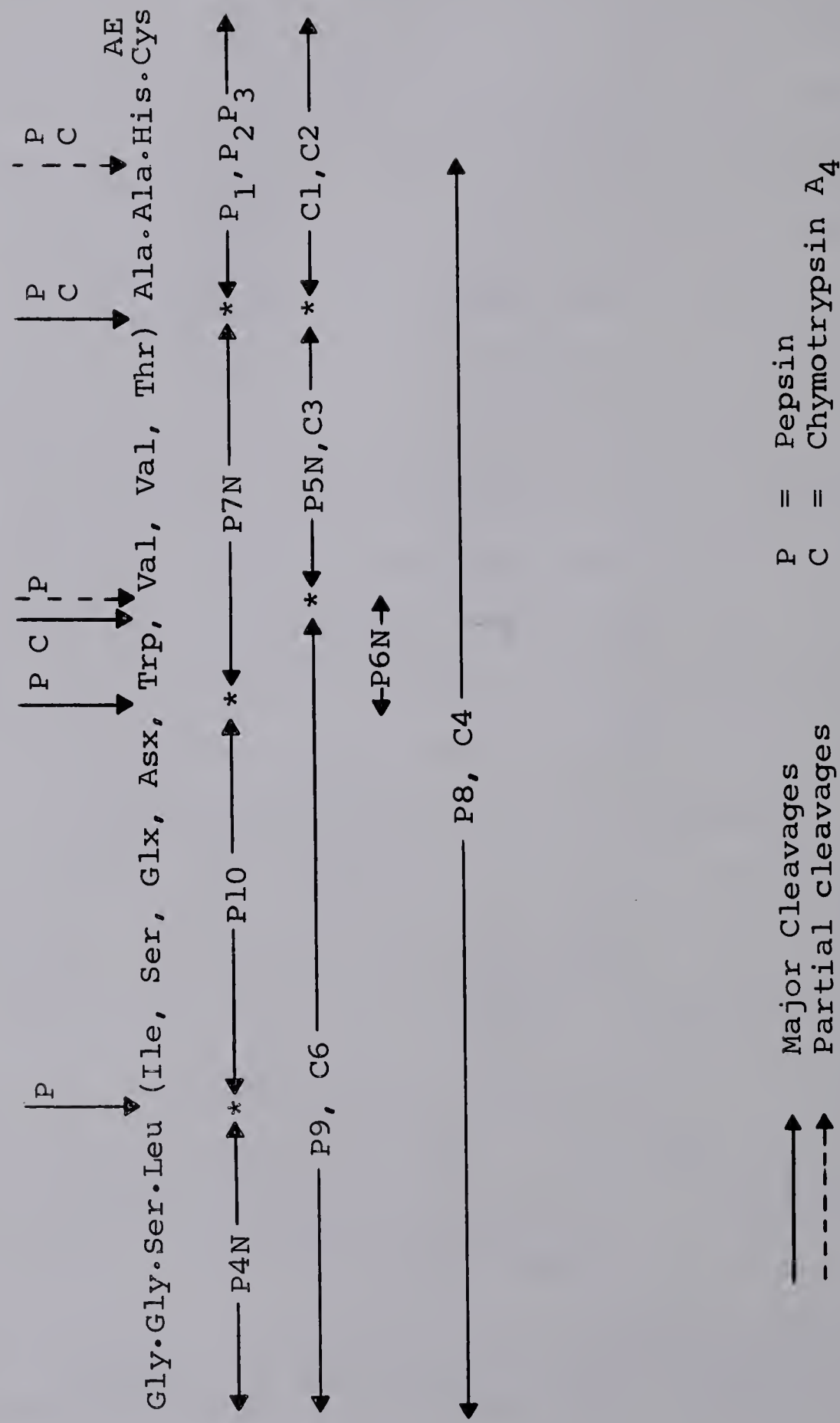
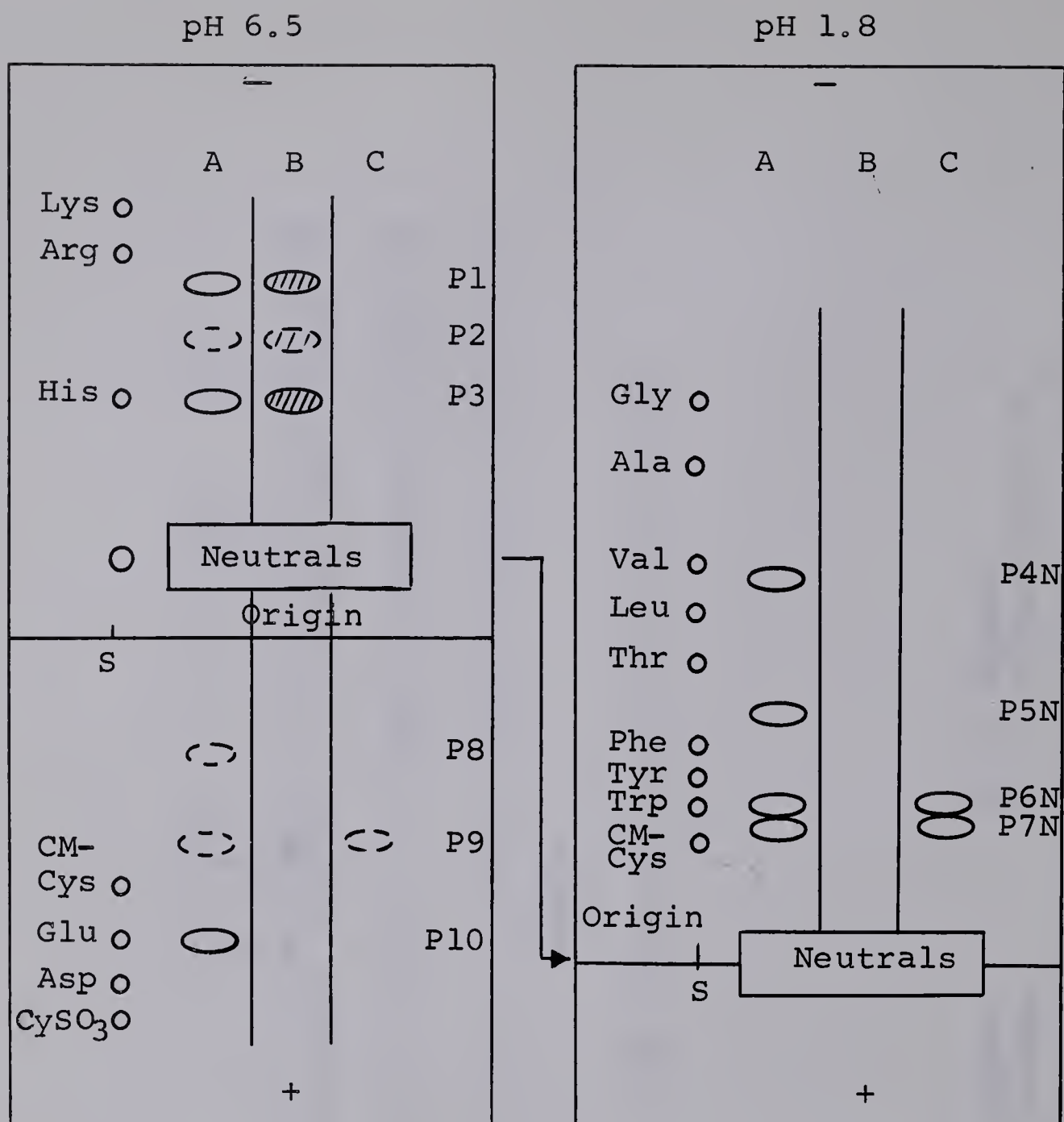


Figure 9. Chymotryptic and peptic peptides from the "histidine-loop"



A = Cadmium-ninhydrin
 B = Pauly reaction (histidine)
 C = Ehrlich reaction (tryptophan)
 S = Standard amino acids

Figure 10. Ionophoresis at pH 6.5 and pH 1.8 of peptic peptides from the "histidine-loop"

quantities was substantiated by the absence of an Ehrlich test for tryptophan. The dipeptides ALA-ALA and HIS-AE.CYS, which would be liberated by partial cleavage, were not detected on the chromatograms. Since peptide C4 is acidic in nature one can reasonably assume that it is void of basic groups.

c. Peptic Digestion of the "Histidine-Loop" Peptide

(i) Purification of Peptides

To 0.35 μ moles of the histidine peptide^{*}, 2 mls of 5% formic acid and 230 μ l of a pepsin stock solution (5.2 mg. per ml of 5% formic acid) were added, and the digest was incubated overnight at 37°. The molar ratio of the "histidine-loop" peptide/pepsin was 50/1. The digest was spotted at a concentration of 0.01 μ moles per cm in the center of a Whatman #1 paper. Standard amino acid mixtures of S1 and S2 were run in parallel. Ionophoresis was conducted at pH 6.5, 50 volts/cm for fifty minutes. Initially only the standard amino acids were developed with ninhydrin in order to detect the neutral band. This band was subsequently seen 10 cm from the anode of a Whatman #1 paper and subjected to pH 1.8 ionophoresis at 80 volts/cm for twenty minutes. The results of staining side strips of the pH 1.8 and 6.5 ionograms with detection reagents are shown in Figure 10.

The acidic, neutral and basic peptides were eluted with water, 0.01N NH_4OH and 0.01N acetic acid, respectively. Suitable aliquots were removed for N-terminal and amino-acid analyses. The results are reported in Table 4.

* Additional material purified as outlined (Section 4b)

TABLE IV

N-Terminal and Amino Acid Analyses of Peptic Peptides from
the "Histidine-loop"

<u>Peptide</u>	<u>N-Terminal</u>	Composition									
		AE									
		His	Cys	Asp	Thr	Ser	Glu	Gly	Ala	Val	Ile Leu Trp
P1	Ala	0.8	0.6						2.0 ^a		
P3	Ala	1.0	0.4		0.4				2.0 ^a		
P4N	Gly			0.5	0.3	1.1	0.2	2.0 ^a	0.4	0.1	1.0
P7N	Trp				1.1				2.0 ^a		+++
P10	Ile			1.0 ^a		0.9	1.0			1.0	

a. Arbitrarily taken as reference standard

Partial peptic cleavages are indicated by the presence of minor bands P5N, P6N, P8 and P9 on the ionograms as shown in Figure 10. Based on their amino acid compositions, electrophoretic mobility and color reactions, the above mentioned peptides could be arranged with the major peptides (Table 4; P1, P3, P4N, P7N and P10) as presented in Figure 9.

(ii) Sequence Determination

The sequence of peptides P1, P3 and P4N was known previously (16, 17), therefore only peptides P7N and P10, comprising the centre portion of the "histidine-loop", were subjected to the Dansyl-Edman sequential analysis.

Conventional analysis could not be applied to P7N due to a suspected N-terminal tryptophan residue. An alternative approach, utilizing CHT-A₄ or carboxypeptidase A was suggested by the work of Gray (212). It was decided that chymotryptic digestion of the dansylated peptide would be the most favourable approach.

To 0.04 μ moles of the freeze-dried peptide P7N, 60 μ l of 0.1 M NaHCO₃ was added and the solution was taken to dryness. The residue was dissolved in 60 μ l of water, 60 μ l of dansyl chloride (2.5 mg/ml of acetone) was then introduced and dansylation was carried out at 37° for three hours. Subsequent to evaporation of the acetone-water, the residue was redissolved in 50 μ l of water. Chymotryptic digestion was initiated by adding 25 μ l of a stock solution of enzyme (0.8 mg/ml H₂O) and the weakly alkaline digest was incubated at 37° overnight. The liberation of DNS-TRP in high yields was

Relative Mobility ($\mu_{Asp}^{6.5}$)

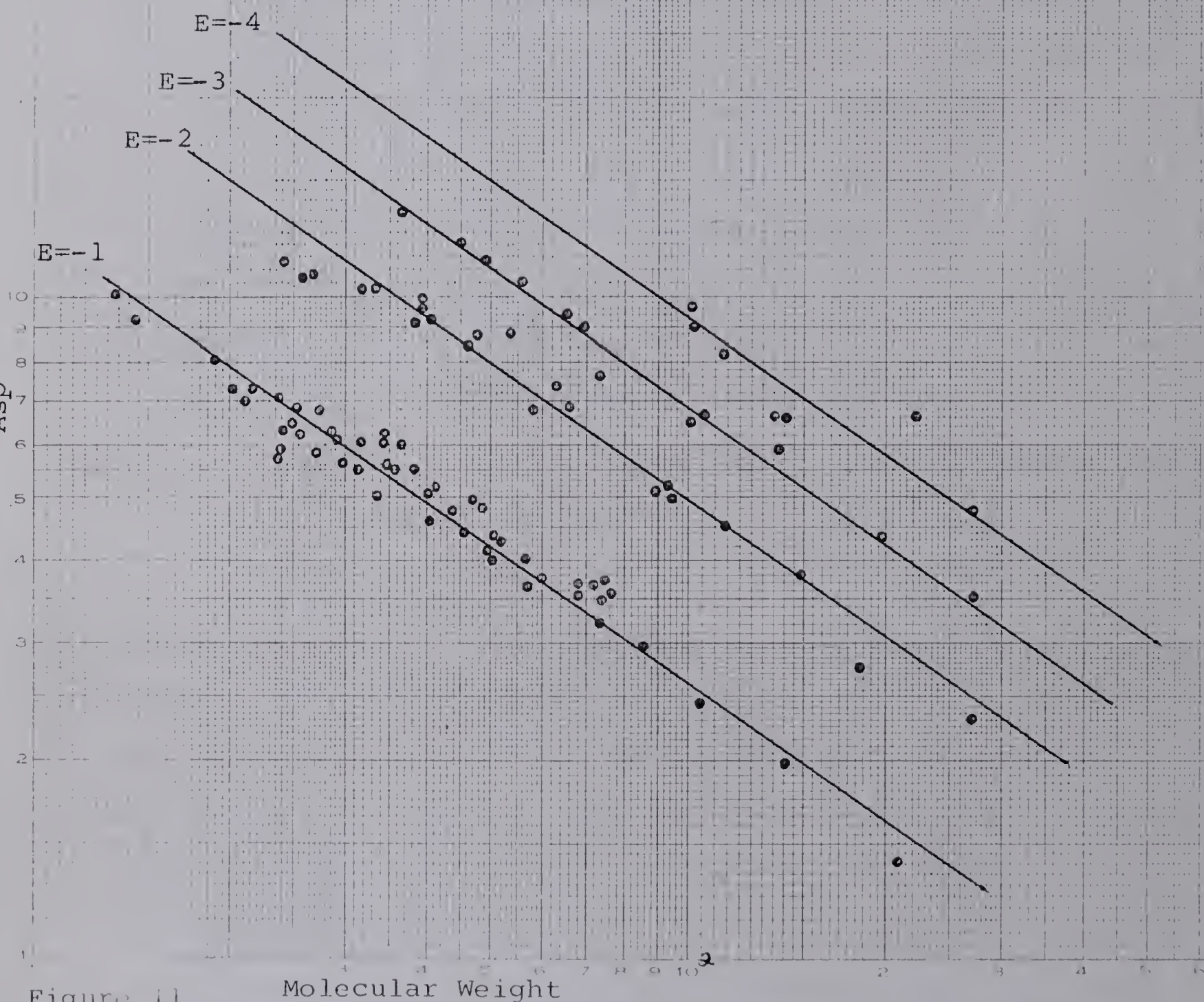


Figure 11. Molecular Weight

A plot of the peptide mobility (μ), relative to free aspartic, versus molecular weight. Measurements were made from the midpoint of the neutral amino acid spot which compensated for endosmosis and variations in the position of the origin. Ionophoreses were carried out at 50 volts/cm in tanks (pH 6.5) described in Appendix A. The majority of the runs involved the peptide moving 10 cm or more. (Offord (217)).

shown upon ionophoresis at pH4.38 by running an aliquot of the digest in parallel with authentic DNS-TRP.

The remaining digest (55 μ l) was spotted as a 3 cm band (about 0.01 μ moles/cm) at a distance of 10 cm from the anode on a Whatman #1 paper. Ionophoresis at pH1.8, 80 volts/cm for 20 minutes revealed the presence of a weak ninhydrin band slightly in advance of phenylalanine. The mobility of this fragment of P7N (designated P7N CHT) was identical to that of a chymotryptic peptide isolated previously (C3; VAL, VAL, THR). Peptide P7N CHT was eluted with 0.01 N NH_4OH , freeze-dried and taken up in 200 μ l of pyridine-water. Dansyl-Edman sequence determination, as outlined earlier, confirmed the sequence of P7N CHT to be VAL-VAL-THR. Based on the accumulated data, peptide P7N can unambiguously be assigned the structure TRP-VAL-VAL-THR .

Elucidation of the amino acid sequence in peptide P10 (ILE,SER,GLX,ASX) demanded not only the order of the residues, but also a knowledge of whether or not aspartic acid or glutamic acid existed as an amide. Based on the mobility at pH6.5 ionophoresis, at least one or possibly both residues were present as the free acid. R.E. Offord (217) has recently devised a simple method whereby the net charge on a peptide at pH6.5 can be determined graphically. The log of the relative mobility of a peptide, with respect to aspartic acid, was plotted against the logarithm of the molecular weight. The data was found to coincide with one of a series of parallel lines depicting a net charge of -1,-2, -3 or -4 (Figure 11). All measurements were taken from the neutral

TABLE V

Dansyl-Edman Sequence Determination of the Peptic Peptide P10

<u>Edman Step</u>	<u>Peptide</u>	<u>N-Terminal</u>	<u>Sequence</u>
0	Ile, Ser, Glx, Asx	Ile	→ Ile (Ser, Glx, Asx)
1	Ser, Glx, Asx	Ser	→ Ile · Ser (Glx, Asx)
2	Glx, Asx	Glu	→ Ile · Ser · Glx (Asx)
3	Asx	Asp ^a	→ Ile → Ser → Glx → Asp

a. Before and after acid hydrolysis.

region so as to offset endosmosis. The validity of the plot has been attested to through numerous applications in the laboratory of Dr. B.S. Hartley, Cambridge, England and in our own laboratory. Peptide P 10 possessed a relative mobility of 0.79 which corresponded to a net charge of -2 on graphical analysis. The existence of aspartic and glutamic as free acids was suggested.

Dansyl-Edman sequence determination of P10 is summarized in Table 5. Based on the data presented, the sequence may be written as ILE.SER.GLX.ASP. Following the third Edman step the N-terminal residue was identified as aspartic acid before and after acid hydrolysis, thus demonstrating the absence of asparagine.

Evidence was still required to unequivocally demonstrate the presence of glutamic acid and not glutamine. Mild enzymic digestion of P10 followed by characterization of the products on pH 6.5 ionophoresis was employed. The GLX-ASP bond proved to be particularly resistant to pronase (Calbiochem. B Grade) while subtilisin (Nagarse, Teikatu Chemical Co. Ltd., Japan) had no detectable effect on the peptide. Aspartic and glutamic acid isolated from a carboxypeptidase (Worthington) digest, offered the first tangible evidence. Confirmation of the presence of glutamic acid was acquired from a leucine aminopeptidase (LAP) digest. Ten μ l of 0.2 M N-ethyl morpholine pH 8.0, 5 μ l of 0.016 M $MgCl_2$ and 5 μ l of a stock LAP solution (Worthington, 6.2 mg/ml) were added to 0.012 μ moles of peptide P 10 in 20 μ l of water. The digest was incubated at 37° overnight and analyzed qualitatively. Four bands were

clearly seen on the pH6.5 ionogram:

(a) the neutral band containing Ile and Ser

(b) free aspartic acid

(c) free glutamic acid

and (d) an acidic band in advance of glutamic acid.

Application of the graphical charge determination method of Offord (217) (Figure 11) indicated the acidic band had a net charge of -2 and a mobility, relative to aspartic acid, of 1.13. The peptide was undoubtedly GLU-ASP. Resistance of the GLU-ASP bond to LAP hydrolysis has also been observed by Ingram (218).

6. Discussion

Elucidation of the sequence of the "histidine-loop" has revealed peculiarities in the substrate specificity of CHT-A₄ and has provided an insight into the chemistry of AE-cysteine residues.

Perhaps the most surprising observation was the facility with which chymotrypsin hydrolysed a THR-ALA bond while only partially hydrolysing a "specific" tryptophan bond. A plausible explanation must go beyond the particular amino acid side chain and invoke residues in proximity to the susceptible bond. The presence of glutamic acid and aspartic acid N-terminal to the tryptophan residue were undoubtedly responsible for the partial cleavage of the TRP-VAL peptide bond. These acidic residues could conceivably disrupt the orientation of the susceptible bond in the active site of chymotrypsin.

A similar situation existed in the sequence ASP-ASP-PHE-ALA-ALA, residues 128-132 of CHTG-A, where the PHE-ALA bond was not extensively hydrolyzed by chymotrypsin (11). Moreover, Schroeder and Jones (190) have observed that the presence of an aspartic acid residue, N-terminal to lysine, markedly decreases tryptic hydrolysis.

The lability of the THR-ALA peptide bond appears to be due to the influence of the adjacent amino acids which are non-polar in nature. Enhancement of chymotryptic hydrolysis of a THR-X bond has been shown to occur in the presence of a valine residue N-terminal to threonine (11). Observance of this "non-specific" chymotrypsin split confirms earlier suspicions that fragmentation of the "histidine-loop" peptide during tryptic digestion of S-(β -aminoethyl)-CHTG-B was due to contaminating chymotrypsin.

The basic peptides from peptic (P1, P2, P3) and chymotryptic (C1, C2) digests of the "histidine-loop" were found to be identical. Comparison of the strongly basic P1 and C1 peptides with the less basic P3 and C2 peptides revealed consistently lower recovery of AE-cysteine from acid hydrolysates of the latter. Oxidation of the sulfur atom to the sulfone, in the side chain of AE-cysteine could account for the multiple bands. An increase in molecular weight coupled with a possible depression of the pK_2 , through field effects of the sulfone, could lead to the altered mobilities. Peptides

P1 and C1 thus contain AE-cysteine in its unoxidized form — a structure which is stable to acid hydrolysis (Appendix D). The trace amounts of peptide P2 likely represent the AE-cysteine side chain in its intermediate sulfoxide form ($-\overset{\text{O}}{\underset{\text{O}}{\text{S}}}-$). Major bands P3 and C2 yielded low recoveries of AE-cysteine upon acid hydrolysis. The sulfone ($-\overset{\text{O}}{\underset{\text{O}}{\underset{\text{O}}{\text{S}}}}-$) present in these bands is thought to be unstable and to decompose into taurine and serine. This theory, although plausible, cannot account for the apparent recovery of the native AE-cysteine from the sulfone derivative unless the two species are not separable on the analyzer. Whatever the alteration in the structure of the peptide that causes the multiple bands, the side chain of AE-cysteine is certainly involved.

The "diagonal" paper ionophoretic technique of Brown and Hartley (132) coupled with redirection of the tryptic hydrolysis to cystine or cysteine bonds through formation of AE-cysteine residues, is a powerful tool in the establishment of overlap peptides for sequence study. The reagent of choice for aminoethylating cysteine residues is ethylenimine-acetate buffer at pH 8.0.

Goldberger and Anfinsen (219) have selectively masked the ϵ -aminogroups of lysine with ethyl trifluoroacetate, thereby directing tryptic hydrolysis solely to the arginine residues. S-(β -aminoethyl)-cysteine would be expected to react in a similar manner to lysine. Removal of the trifluoroacetyl moiety (TFA) from the ϵ -amino groups with M piperidine at 0° exposes the tryptic sensitive sites.

Application of the technique introducing AE-cysteine residues into a protein will be one of the most useful tools for future sequence elucidation. Manipulation of the TFA blocking group selectively to lysine (220), S-(β -aminoethyl)-cysteine, or both and application of an arginine blocking group, 1,2 cyclohexanedione (221), can yield a variety of overlapping peptides from tryptic digests.

The role of the "histidine-loop" in chymotrypsin and trypsin has been postulated as the major substrate binding site by Bender, Killheffer and Kédzy (151,152). Their hypothesis was based on extensive structural homologies existing in this region and on the identity of the deacylation rate of non-ionic acyl-trypsins and acyl-chymotrypsins over a 10^5 -fold range of rate constants. It is well known that trypsin exhibits a high degree of specificity towards lysine and arginine residues. Support for the presence of an anionic site and a hydrophobic slit to accommodate these amino acids in the active site has been recently obtained. (157-160) Although the "histidine-loop" in trypsin is hydrophobic in nature it does not, unfortunately, contain an anionic group to fulfil the binding site requirements. In order to compensate for this deficiency, Bender et al. (152) have postulated the presence of an auxiliary anionic residue. In contrast, the "histidine-loop" in CHT-A₄ has been shown to contain an acidic residue in an otherwise hydrophobic sequence. This residue would not be expected to facilitate the binding of an aromatic group to the "histidine-loop". Although the "histidine-loop" of CHT-A₄ and trypsin do not entirely complement the structures

Chymotrypsinogen B

40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59
His	Phe	Cys	Gly	Gly	Ser	Leu	Ile	<u>Ser</u>	<u>Glu</u>	<u>Asp</u>	Trp	Val	Val	Thr	Ala	Ala	His	Cys	Gly
		S																S	

Chymotrypsinogen A

42																			58
His	Phe	Cys	Gly	Gly	Ser	Leu	Ile	<u>Asn</u>	<u>Glu</u>	<u>Asn</u>	Trp	Val	Val	Thr	Ala	Ala	His	Cys	Gly
		S																S	

Trypsinogen

42																			58
His	Phe	Cys	Gly	Gly	Ser	Leu	Ile	<u>Asn</u>	<u>Ser</u>	<u>Gln</u>	Trp	Val	Val	Ser	Ala	Ala	His	Cys	Lys
		S																S	

————— Non-homologous regions

Figure 12. Comparison of the amino acid sequence of the "histidine-loop" in CHTG-B, CHTG-A₄ and trypsinogen.

of their respect substrates, one cannot eliminate the possibility that part of the "histidine-loop" could be involved in a substrate binding site.

In comparison to the "histidine-loops" of CHTG-A and trypsinogen, the "histidine-loop" of CHTG-B was found to be the most hydrophilic in nature. (Figure 12). Extensive homologous structures exist in the "histidine-loops" of these enzymes with the notable exception of the short sequence, residues 48 to 50, where variation in amino acid sequence and in the nature of the amino acid is permitted. In CHTG-A and particularly CHTG-B, the sequences ASN-GLU-ASN and SER-GLU-ASP respectively, located midway between the half-cysteine residues 42 and 58, disrupts the predominantly hydrophobic nature of the "histidine-loop". The corresponding sequence in trypsinogen ASN-SER-GLN does not markedly alter the hydrophobicity of the "histidine-loop". The role of residues 48 and 50 is likely to link the hydrophobic "arms" of the "histidine-loop" and thus impart rigidity to the structure. The stereochemical relationship between the two histidines adjacent to the disulfide bridge is thus preserved.

Studies in this laboratory have revealed that CHT-B is resistant to inhibition by DFP, DPCC and L-TPCK when compared to CHT-A₄. (13,129, Chapter III). If the hydrophobic "arms" of the "histidine-loop" comprise a portion of a binding site on the enzyme surface, then the presence of two acidic residues at positions 49 and 50 in CHT-B would be sufficiently near to influence the binding of substrates or inhibitors.

Binding Site 1

	95	96	97	98	99	100	101	102	103	104	105	106	107
Trypsin	Asn	Pro	Leu	Thr	Asn	Asn	Asn	<u>Asp</u>	Ile	Met	Leu	Ile	Lys
CHT-A ₄	Asn	Ser	Leu	Thr	Ile	Asn	Asn	<u>Asn</u>	Ile	Thr	Leu	Leu	Lys
CHT-B	Ser	Ile	Leu	Thr	Val	Arg	Asn	<u>Asp</u>	Ile	Thr	Leu	Leu	Lys

Binding Site 2

	160	161	162	163	164	165	166	167	168	169	170	171	172
Trypsin	Ala	Pro	Ile	Leu	Ser	<u>Asp</u>	Ser	Ser	Cys	Lys	Ser	Ala	Tyr
CHT-A ₄	Leu	Pro	Leu	Leu	Ser	<u>Asn</u>	Thr	Asn	Cys	Lys	Lys	Tyr	Trp
CHT-B	Leu	Pro	Ile	Val	Ser	<u>Asn</u>	Thr	Asp	Cys	Arg	Lys	Tyr	Trp

Binding Site 3

	181	182	183	184			185	186	187	188	189	190	191
Trypsin	Phe	Cys	Ala	Gly	Tyr	Leu	<u>Glu</u>	Gly	Gly	Lys	Asn	Ser	Cys
CHT-A ₄	Ile	Cys	Ala	Gly	<u>Ala</u>	Ser	Gly	Val	Ser	Ser	Cys
CHT-B	Ile	Cys	Ala	Gly	Ala	Ser	Gly	Val	Ser	Ser	Cys

Figure 13. The amino acid sequence of possible substrate binding sites in CHT-A₄, CHT-B and trypsin.

It is possible that the observed decrease in the susceptibility of CHT-B to the above mentioned inhibitors may be due to the presence of these acidic residues. In addition, these residues may contribute to the increase in the deacylation rate of non-ionic acyl-CHT-B (anisoyl-, trimethylacetyl- and cinnamoyl-) when compared to the correspondings acyl-derivatives of CHT-A₄. Although these deductions are speculative at this time, the significance of the "histidine-loop" in the enzymes studied suggests that the acidic residues in CHT-B cannot be disregarded.

Smillie and Hartley (189) have suggested that additional regions in the CHT-A₄ and trypsin could participate as substrate binding sites. Attention was directed towards homologous, predominantly hydrophobic, sequences in which an acidic residue present in trypsin was absent in CHT-A₄. Such a substitution in trypsin introduces a negative charge into an otherwise non-polar sequence thereby partially fulfilling the requirements of the binding site. The regions considered are between residues 95 and 114, between 162 and 170 and between 181 and 194. (Figure 13) For comparison, the sequences corresponding to these regions in CHT-B are included. From the studies on CHT-B the postulated binding site encompassing residues 95 to 114 may be rejected since the aspartic acid residue found at position 102 of trypsin is also found to be present in CHT-B. The two additional possibilities have been supported by sequence studies on CHT-B.

The nature of the substrate binding site in CHT-A₄ may be a hydrophobic depression on the enzyme surface formed by

segments of polypeptide chains likely involved in the serine "knot". It should be noted that the binding site sequences present in Figure 13 (residues 160 to 172 and 181 to 191) possess half-cystine residues. These particular residues (168 and 191) are known to form intrachain disulfide bridges in the C-chain with half-cystine residues 182 and 221, respectively. (132) Thus, these disulfide bridges, in addition to the interchain disulfide bridge between the B and C chains (residues 136 and 201), comprise the so-called serine "knot". (11) The postulated substrate binding regions, therefore, appear to be particularly attractive candidates.

Numerous studies have unequivocally demonstrated the proximity of methionine-192 to the active site of CHT-A₄ (42, 47, 48, 76, 88, 91, 92, 156). Transformation of the hydrophobic thioether side chain of methionine into a hydrophilic side chain, through oxidation to the sulfoxide or alkylation to yield a sulfonium salt, has been shown to be associated with an increase in the Michaelis constant without altering the rate constant k_3 . Thus methionine-192 appears to associate with a hydrophobic region involved in substrate binding.

Neurath and Hartley (69) have postulated that two tryptophan residues form a "hydrophobic slit" to accommodate the aromatic side chains of phenylalanine, tyrosine and tryptophan which are separated from the asymmetric carbon by a methylene bridge. The observations of Abrash and Nieman (70) and Almond et al. (71) that dimethyl substitutions on the β -carbon of ATEE completely prevented hydrolysis of the chymotryptic

substrate lend support to the "hydrophobic slit" hypothesis. The proximity of one or two tryptophan residues to the active site of CHT-A₄ was suggested by the studies of Dixon and Schachter (76). ATEE in the presence of hydrogen peroxide caused the oxidation of methionine 180 and 192, 1 to 2 residues of tryptophan and 1 to 2 residues of half-cystine. The tryptophan residue(s) destroyed were not identified, but it appears likely that residues 172, 207 and 216 may have been involved since these residues are in the neighbourhood of the serine "knot" disulfide bridges. Tryptophan-172 is particularly interesting since it is a component of the "methionine-loop" - a homologous structure identified in several "serine" enzymes (12,14).

III. KINETIC STUDIES ON THE INHIBITION OF THE CHYMOTRYPSINS WITH BIFUNCTIONAL REAGENTS.

1. Introduction

Knowledge of the kinetics of a reaction between an enzyme and an inhibitor is a necessary prerequisite before structural studies are undertaken. The attainment of rapid inhibition with a minimum of side reactions usually necessitates the manipulation of numerous parameters before suitable conditions are obtained.

Preliminary studies on the effect of pH on the inactivation of CHT-A₄ and CHT-B with various bifunctional reagents were carried out in order to obtain optimum conditions for the inactivation of the enzymes. Structural studies designed to elucidate the mode of inhibition by the bifunctional reagents were conducted on the inactivated enzyme prepared according to the preliminary pH studies.

A more definitive study on the rate of inactivation of CHT-A₄ was performed with two reagents (L-1-tosylamido-2-phenylethyl chloromethyl ketone, L-TPCK; and phenoxymethyl chloromethyl ketone, PMCK) whose modes of inactivation had been delineated. The results obtained with L-TPCK and PMCK were not sufficiently encouraging and informative to merit such a detailed kinetic study of the other reagents employed in this research. The bifunctional reagents investigated in the present studies include: L-TPCK ($\text{C}_6\text{H}_5 \cdot \text{CH}_2 \cdot \text{CH}(\text{NH}\text{SO}_2\text{C}_7\text{H}_7) \cdot \text{CO} \cdot \text{CH}_2\text{Cl}$); PMCK ($\text{C}_6\text{H}_5 \cdot \text{O} \cdot \text{CH}_2 \cdot \text{CO} \cdot \text{CH}_2\text{Cl}$) and a homologous

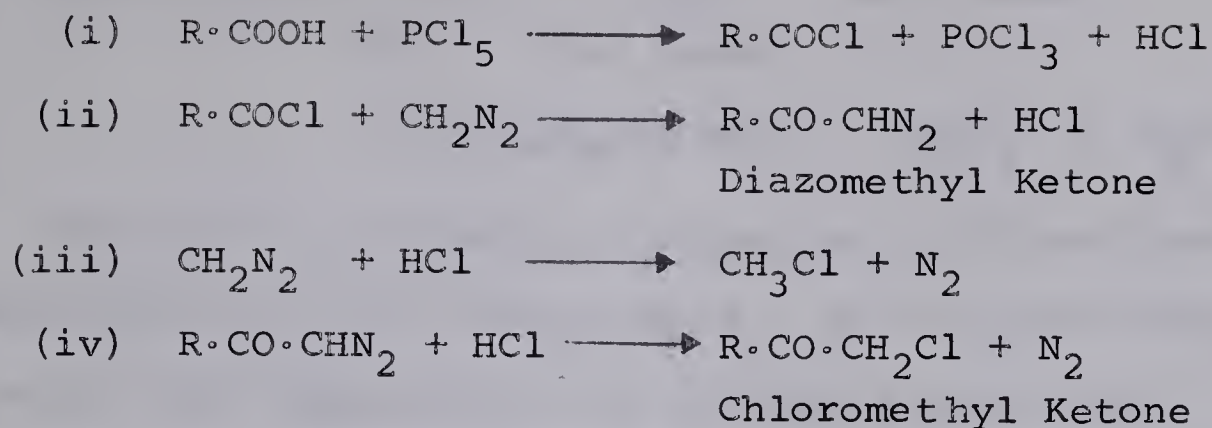
series of phenylalkyl chloromethyl ketones ($C_6H_5 \cdot [CH_2]_n \cdot CO \cdot CH_2Cl$, where $n = 0, 1, 2$). Reagents comprising the homologous series were chloroacetophenone (CA, $n = 0$), benzyl chloromethyl ketone (BCK, $n = 1$), and β -phenylethyl chloromethyl ketone (β PECK, $n = 2$). The common chemical names were used for the first two reagents in the series in order to exclude ambiguous abbreviations even though the classical nomenclature - phenyl chloromethyl ketone ($n = 0$), and phenylmethyl chloromethyl ketone ($n = 1$) - was more descriptive.

Earlier studies by Enenkel (129) and Parkes (13) in this laboratory revealed that CHT-B was inhibited by DFP and diphenyl carbamyl chloride at a much slower rate than was CHT-A₄. The bifunctional reagents synthesized by this worker offered an opportunity to investigate this apparent resistance of CHT-B to inhibition. Furthermore, a study of the effect of pH on inhibition and the second - order rate constants of inhibition of the chymotrypsins with these reagents enable a comparison of CHT-A₄ and CHT-B on a kinetic basis.

2. Methods

The bifunctional reagents to be discussed in this thesis were synthesized in a similar manner utilizing the Arndt-Eistert reaction. The transformation of a carboxylic group ($R \cdot COOH$) into a chloromethyl ketone group ($R \cdot CO \cdot CH_2Cl$) was the common synthetic pathway involved. The most direct synthetic method available is outlined below and involves the use

of a highly toxic gas-diazomethane (CH_2N_2)

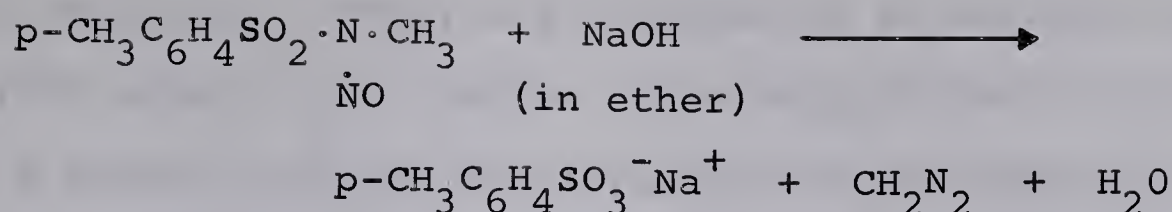


The starting material for the synthesis was either the acid or the acyl chloride depending on whether or not the latter was commercially available. The intermediate diazomethyl ketone could be isolated if the acyl chloride was slowly added to an excess of CH_2N_2 . When the reagents were added in this manner reactions (ii) and (iii) would predominate. Formation of the chloromethyl ketone (reactions (ii) and (iv)) was promoted by adding the reagents in the reverse order, that is, the diazomethane was added to an excess of the acyl chloride. The HCl liberated in reaction (ii) could in turn react with the diazomethyl ketone thus leading to the chloromethyl ketone via reaction (iv). Additional anhydrous hydrogen chloride gas destroyed the excess CH_2N_2 and converted any remaining diazomethyl ketone to the chloromethyl ketone.

Excellent reviews dealing with the chemistry and synthetic application of diazomethane have been written by Eistert (226) and Bachmann and Struve (227).

a. Preparation of Diazomethane (CH_2N_2)

Diazomethane was prepared using N-methyl-N-nitroso-p-toluenesulfonamide as the source of the reagent.



Alternative methods utilizing the toxic and unstable nitro-somethylurea were not satisfactory. Gold dilute ethereal solutions were employed in all synthetic procedures.

The exact quantity of CH_2N_2 present could be readily determined by reacting an aliquot of the ethereal diazomethane solution with an accurately measured excess of benzoic acid in ether.



The remaining benzoic acid was titrated with dilute sodium hydroxide using phenolphthalein as indicator. Due to the toxic nature of the reagent, all manipulations were performed in a fume hood equipped with an efficient exhaust system.

b. Synthesis of Bifunctional Reagents*

N-Tosyl-L (and D)-phenylalanine, prepared from L (and D)-phenylalanine and p-toluenesulfonyl (tosyl) chloride, were transformed into their crystalline acyl chlorides by reaction with phosphorous pentachloride. These acyl chlorides, in addition to phenoxyacetyl chloride, hydrocinnamoyl chloride, phenylacetyl chloride and anisoyl chloride, were converted to their respective chloromethyl ketones (L-TPCK, D-TPCK, PMCK, β PECK, CA, ACK) by the reaction with a molar ratio of $\text{CH}_2\text{N}_2/\text{R COCl}$ of 2/1 followed by treatment with dry hydrogen gas. L-1-N-methyl-N-tosylamido-2-phenylethyl chloromethyl

*

See Appendix D for detailed description of chemical synthetic procedures.

ketone (N-methyl-L-TPCK) was prepared in an analogous manner to L-TPCK except that a molar ratio $\text{CH}_2\text{N}_2/\text{R}\cdot\text{COCl}$ of 9/1 was used in order to secure N-methylation of the tosylamido group. α -chloroacetophenone (CA) was purchased from Eastman Organic Chemicals.

Since these reagents are potent skin irritants, care was exercised during their handling.

The purity of the preparations was normally based upon two criterion: (1) the agreement between the observed melting point and the melting point reported in the literature, and (2) elemental analyses. In addition to these criterion, L-TPCK and D-TPCK were further characterized by comparing their molar optical rotatory dispersion spectra.

It should be noted that L-TPCK prepared in this laboratory was found to be contaminated with what appeared to be L-1-tosylamido-2-phenylethyl hydroxymethyl ketone (L-TPHK, $\text{C}_6\text{H}_5\cdot\text{CH}_2\cdot\text{CH}(\text{NHSO}_2\text{C}_7\text{H}_7)\cdot\text{CO}\cdot\text{CH}_2\text{OH}$). Formation of L-TPHK was attributed to a sodium bicarbonate washing of the ether-HCl solution of L-TPCK at the completion of the synthesis. Bicarbonate washings were avoided in the synthesis of D-TPCK and other chloromethyl ketones.

Kinetic studies reported in this chapter were performed on an authentic sample of L-TPCK purchased from Sigma Chemical Company. Comparison of the Sigma L-TPCK and D-TPCK, synthesized in this laboratory, was performed by Dr. C.M. Kay using a Cary 60 Automatic Recording Spectropolarimeter. The reagents were found to be true isomers, being equal in rotation and opposite in sign.

Structural investigations reported in Chapter IV were conducted with CHT-A₄-TPCK and CHT-B-TPCK inhibited by L-TPCK prepared in this laboratory. L-TPHK, being chemically unreactive, would not have any influence on the structural studies reported.

c. Rate Assay of Chymotrypsin

Enzyme activity was determined by a "rate or efficiency" assay using an excess of a synthetic substrate N-acetyl-L-tyrosine-ethyl ester (ATEE) (Mann Research Laboratories) (167). Assays were routinely performed using a pH-stat comprised of a Radiometer TTT1a titrator coupled to a Radiometer Titragraph type SBR2C (Copenhagen), and a type SBULa syringe burette. The chymotryptic ester hydrolysis was carried out in a thermostated reaction vessel designed for small volumes into which a single stem Radiometer GK202IC electrode was inserted (168). An aliquot (2.5 mls) of 0.01M ATEE in 0.01M tris.HCl buffer, pH8.0, containing 0.01M KCl and 0.02M CaCl₂ was used for enzyme assay at 25°. Calcium and potassium ions were included since they are known to stabilize the chymotrypsins (13,169,170). The hydrolysis of ATEE was initiated by introducing 100 µls of a dilute chymotrypsin-A₄ (or CHT-B) solution (1.6×10^{-6} M). The proton resulting from the ionization of N-acetyl-L-tyrosine was titrated automatically at pH8.0 with 0.2M NaOH. Setting the chart speed of the recorder at 10 mm/min yielded a 45° tracing representing a base uptake of approximately 8 µls/min. Under these conditions a linear tracing was obtained for the seven minute duration of the assay.

Alkali uptake could vary from 4 to 11 $\mu\text{l}/\text{min}$ without seriously affecting the k^1 calculation. Increasing the amount of active enzyme in the assay led to a non-linear response on the recorder depicting a departure from zero-order reaction conditions. Based on the initial slope of the hydrolysis and the milligrams of enzyme nitrogen assayed, chymotryptic activity was expressed as the apparent specific zero-order rate constant k^1 (meq. substrate per ml. hydrolysed per min per mg enzyme nitrogen per ml). k^1 was readily calculated from the relationship:

$$k^1 = \frac{\text{initial slope of hydrolysis in } \mu\text{l}/\text{min}}{\text{mg of enzyme nitrogen/ml}} \times \frac{\text{normality of alkali}}{\text{of alkali}}$$

where mg of enzyme nitrogen was determined from the expression:

$$\% \text{ nitrogen} \times 10 \times \frac{\text{absorbance of aliquot}}{E_{1\text{cm}}^{1\%}} \times \frac{\text{volume of aliquot in ml.}}{\text{of aliquot in ml.}}$$

The % nitrogen and $E_{1\text{cm}}^{1\%}$ of CHT-A₄ were 16.7 and 20.0 respectively at 282 m μ (10, 188), and 16.0 and 18.7 respectively for CHT-B at 280 m μ (20). The k^1 was a measure of the residual enzymic activity.

3. The Effect of Competitive Inhibitors on the Reaction of CHT-A₄ with L-TPCK and PMCK.

(a) Methods

(i) The Inhibition of CHT-A₄ with PMCK in the Absence and in the Presence of β -phenylpropionate

CHT-A₄ (100 μl of a $3.2 \times 10^{-4}\text{M}$ solution) was added to each of three 2.0 ml aliquots of a 0.05M tris-maleate buffer (0.05M in CaCl_2 , pH 7.0, 4.8% in ethanol, 25°), each containing one of the following: (1) 0.005M PMCK; (2) 0.005M PMCK

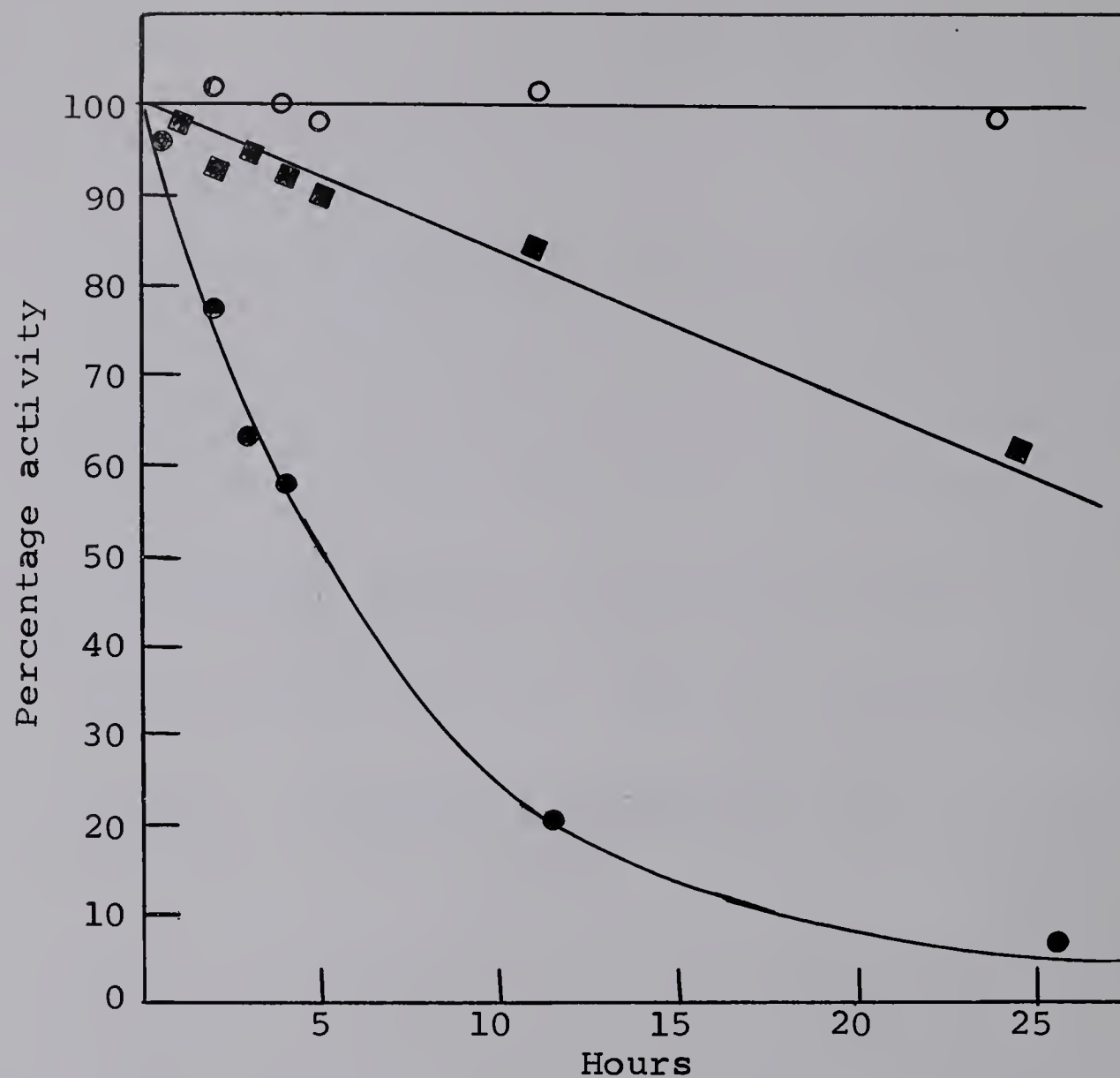


Figure 14. The inactivation of CHT-A₄ by phenoxymethyl chloromethyl ketone (PMCK) in the absence (●—●) and in the presence (■—■) of β -phenylpropionate. Chymotrypsin blank is shown by open circles (○—○).

plus 0.2M β -phenylpropionate and (3) no additional substances. At appropriate intervals, 100 μ l aliquots were removed and diluted in 700 μ l of 5×10^{-3} M HCl at 0°. Residual chymotryptic activity was assayed as described earlier with ATEE. The per cent residual activity was determined.

(ii) The Inhibition of CHT-A₄ with L-TPCK in the Presence of D-TPCK

The incubation solution was composed of a 1.6×10^{-5} M CHT-A₄ solution in 0.05M tris-HCl buffer, 0.05M CaCl₂, pH 7.2, containing 1.6×10^{-4} M L-TPCK, 1.6×10^{-4} M D-TPCK and 200 μ l of ethanol (4%). Aliquots were removed at 5 minute intervals over a 40 minute period, diluted in cold HCl and assayed against ATEE. The apparent first-order rate constant was obtained from a plot of log residual activity versus minutes.

(b) Results and Discussion

The protection of CHT-A₄ against PMCK by the competitive inhibitor β -phenylpropionate (185) is demonstrated in Figure 14. Following a 24 hour exposure to PMCK, CHT-A₄ possessed only 7% of the activity of the control. β -phenylpropionate, which is known to combine reversibly with the active site of CHT-A₄ ($K_I = 0.45 \times 10^{-2}$ M), effectively retarded the inactivation of the enzyme by PMCK. The data support the contention that PMCK is truly bifunctional in nature and is bound to the active site prior to alkylation of histidine-57.

Similar studies by Schoellmann and Shaw (104) have shown that β -phenylpropionate protects CHT-A₄ from inactivation by L-TPCK. Erlanger et al. (33) have demonstrated that indole, a competitive inhibitor (186), completely prevented the inactivation of CHT-A₄ by diphenylcarbamyl chloride.

Earlier studies in this laboratory (13) have shown that β -phenylpropionate and indole are potent competitive inhibitors of CHT-B, $K_I = 1.7 \times 10^{-2}$ and 4.4×10^{-4} M, respectively. Although protection by these reagents has not been demonstrated for CHT-B in the presence of PMCK and L-TPCK, it is undoubtedly analogous with the CHT-A₄ system.

Based on structural studies presented in Chapter IV, β -PECK is known to alkylate residues at the active site of CHT-A₄. In view of these findings and the competitive inhibitor studies just discussed, it is reasonable to conclude that the β -phenylpropionate or indole could successfully protect CHT-A₄ or CHT-B against inactivation by this bifunctional reagent.

Since the D-isomers of chymotryptic substrates are capable of acting as competitive inhibitors, it was expected that D-TPCK could retard the inactivation of CHT-A₄ by the active isomer-L-TPCK. The apparent first-order rate constant for the inhibition of CHT-A₄ by L-TPCK at pH 7.2 was observed to decrease from $9.33 \times 10^{-4} \text{sec}^{-1}$ to $7.70 \times 10^{-4} \text{sec}^{-1}$ in the presence of D-TPCK. Although the D-isomer appears to act as a competitive inhibitor, studies to be presented in Chapter IV demonstrate that D-TPCK is capable of alkylating a methionine residue. Interpretation of the data is therefore not straightforward.

4. The Effect of pH on the Rate of Inhibition of the Chymotrypsins with Bifunctional Reagents

a. Methods

(i) An Approximation of the Effect of pH on the Inhibition of the Chymotrypsins with Bifunctional Reagents

The application of this method was particularly useful for preliminary investigations of the effect of pH on the extent of

inhibition. Although less rigorous than the calculation of the apparent first-order rate constant at specified pH values, the method could, with care, yield reliable data.

CHT-A₄ (or CHT-B) was incubated in a series of buffer at various pH values in the presence and absence of an inhibitor at 25° for one hour (L-TPCK) or for 24 hours with the other bifunctional reagents studied. The buffer systems used exclusively for these experiments were 0.05 M glycine-HCl, pH 3.0; 0.05 M acetate, pH 4.0-5.0; 0.05 M tris-maleate, pH 5.2-8.5 and 0.05 M glycine-NaOH, pH 9.0 (171). All buffers contained 0.05 M CaCl₂. Enzyme blanks were prepared by adding the reagents in the following order: 2.0 mls buffer, 100 μ l of ethanol and 100 μ l of stock enzyme solution (4.8×10^{-4} M in water). Similarly, 2.0 mls of buffer, 50 μ l of ethanol, 50 μ l of a stock inhibitor solution (L-TPCK, 6.4×10^{-4} M; PMCK, 0.13 M; β PECK, 0.063 M; BCK, 0.16 M; CA, 0.064 M in ethanol) and 100 μ l of stock enzyme solution comprised the incubation solution.

Enzyme assays were performed as indicated earlier. A plot of percent inhibition, corrected for autolytic destruction of the chymotrypsins, versus pH revealed the effect of pH on inhibition.

(ii) The Effect of pH on the Rate of Inhibition of CHT-B with L-TPCK

The effect of pH on the rate of inhibition of CHT-B with L-TPCK was obtained using acetate, cacodylate, tris and glycine buffers (171) of 0.05 M concentration, 0.05 M CaCl₂, and 4% in ethanol. Ethanol concentrations of four percent, while not detrimental to the enzyme, were capable of solubilizing sufficient quantities of the inhibitor to give a final inhibitor-to-

enzyme ratio of 10/1. To CHT-B ($1.6 \times 10^{-5}\text{M}$) in 5.0 ml of buffer was added 200 μl of ethanol and the pH was readjusted to the desired value when necessary. A 100 μl aliquot was removed for the blank and added to 700 μl of $5 \times 10^{-3}\text{M}$ HCl at 0° in preparation for enzyme assay. At zero time a 25 μl aliquot of L-TPCK in ethanol ($3.2 \times 10^{-2}\text{M}$), synthesized in this laboratory, was added. Aliquots (100 μl) were removed at suitable time intervals over a period of 90 minutes and diluted in 700 μl of $5 \times 10^{-3}\text{M}$ HCl at 0° . The HCl was sufficiently strong to bring the pH to approximately 3. Assay of the residual CHT-B activity was performed with a suitable amount of the cold HCl solution to yield a base uptake of near 8 $\mu\text{l}/\text{min}$. (45° tracing). The absorbancy value ($A_{280\text{ m}\mu}$), used for the calculation of enzyme nitrogen concentration, was determined from an appropriate dilution of the incubation mixture.

The apparent first-order rate constant was obtained from a plot of log residual activity versus minutes.

(iii) The Effect of pH on the Rate of Inhibition of CHT-A₄ with L-TPCK and PMCK

A more definitive study of the effect of pH on the rate of inactivation of CHT-A₄ with L-TPCK and PMCK, involving the determination of the apparent first-order rate constant (k_{obs}) and the first-order rate constant of the enzyme-inhibitor complex decomposition (k_3), was performed in order to elucidate the nature of the group(s) involved. A single buffer system, 0.05 M tris-maleate (171) containing 0.05 M CaCl_2 , was utilized over the pH range 5.2 to 8.6. The use of the single buffer system was

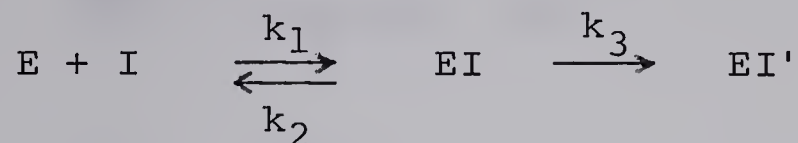
prompted by the observation that phosphate and cacodylate buffers strongly suppressed the inhibition of CHT-A₄ by PMCK. A disadvantage of the tris-maleate buffer was the absorbance of maleic acid at 280 mμ which prevented the determination of enzyme concentration in the presence of the buffer. To circumvent this problem, the enzyme concentration was determined from suitable dilutions of the stock solution.

An aliquot (100 μl) of a stock CHT-A₄ solution (4.8×10^{-4} M in water) was added to 3.0 ml of buffer containing 100 μl of ethanol at 25°. The pH was adjusted, if necessary, to the desired value and a 100 μl aliquot was removed as a blank. A 50 μl aliquot of a stock inhibitor solution (L-TPCK, 1.06×10^{-2} M, Sigma Chemical Co.; PMCK 0.336 M in ethanol) was introduced at zero time. Over a suitable period of time, (L-TPCK, 30 minutes; PMCK, 3 hours) 100 μl aliquots of the incubation were removed and added to dilute solutions of cold HCl. Enzyme assays were performed on suitable aliquots of HCl as outlined earlier.

The determination of the effect of pH on the rate of inhibition of CHT-A₄ by PMCK was complicated by the tardiness of the reaction and by the instability of the inhibitor at alkaline pH. Frequent pH adjustments were necessitated when determining the first-order rate constant at pH 7.5 and 8.0. The apparent first-order rate constant was determined as indicated previously.

(iv) Derivation of Kinetic Relationships

The pathway for the inhibition of the chymotrypsins by the bifunctional reagents is outlined below:



where E is enzyme,

I is the bifunctional reagent,

EI is the non-covalently bound inhibitor-enzyme complex, and EI' is the covalently bound inhibitor-enzyme complex.

Now let total enzyme = E_T

free enzyme = E_F

and
$$K = \frac{(E_F)(I)}{(EI)}$$

and
$$E_T = E_F + EI + EI'$$

$$E_F = E_T - EI - EI'$$

Hence,
$$K(EI) = (E_T - EI - EI') I$$

$$= -I(EI) + I(E_T - EI')$$

$$EI = \frac{(E_T - EI') I}{K + I}$$

Now
$$\frac{d(EI')}{dt} = k_3(EI) = \frac{k_3(E_T - EI') I}{K + I}$$

Now at any given time t, the uninhibited enzyme ($E_T - EI'$) was estimated from ATEE assays so that the velocity of formation of EI', that is $\frac{dEI'}{dt}$ is known.

Now if I is essentially constant during the reaction,
then

$$\frac{dEI'}{dt} = k_{obs}(E_T - EI')$$

where $k_{obs} = \frac{k_3 I}{K+I}$

and in which k_{obs} , the apparent first-order reaction constant, may be calculated from a plot of $\log (E_T - EI')$ against t .

Now if $I \gg K$

CASE A

then $k_{obs} = k_3$.

If $I \ll K$

CASE B

then $\frac{dEI'}{dt} = \frac{k_3}{K} (E_T - EI') I$

The apparent first-order rate constant k_{obs} is equal to $\frac{k_3}{K} I$, and if I is essentially constant ($I \gg E$), the second-order rate constant (k) may be obtained by dividing k_{obs} by (I) to yield $\frac{k_3}{K}$.

If $I \sim K$

CASE C

then, as indicated previously

$$\frac{dEI'}{dt} = \frac{k_3 (E_T - EI') I}{K + I}$$

and $k_{obs} = \frac{k_3 I}{K + I}$.

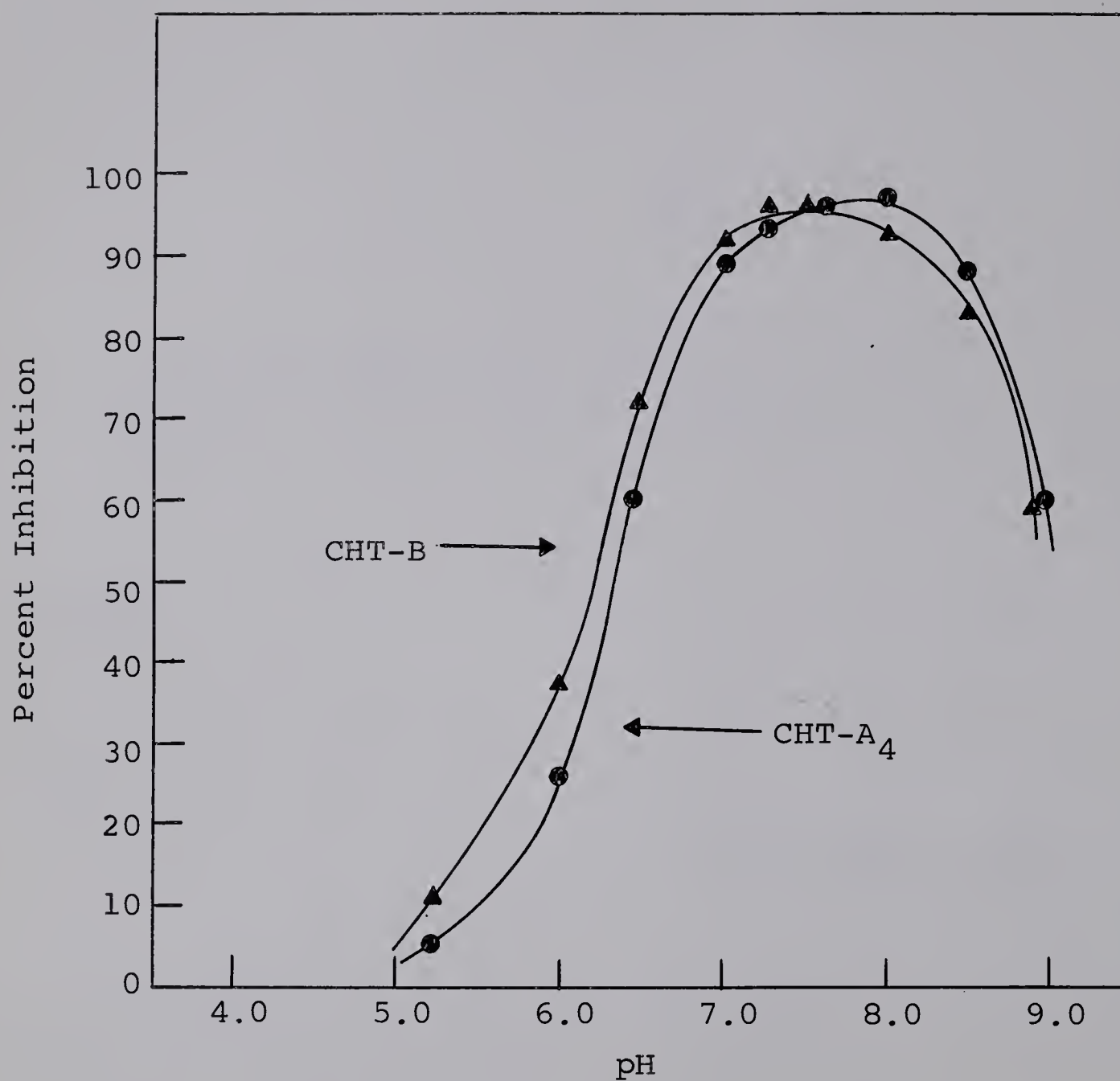


Figure 15: The effect of pH on the inhibition of CHT-A₄ (●—●) and CHT-B (▲—▲) with L-1-tosyl-amido-2-phenylethyl chloromethyl ketone (L-TPCK).

Fig. 1. The dependence of the rate of polymerization on the concentration of the monomer. The reaction was carried out in the presence of 0.01 mole-% of the catalyst. The temperature was 40°C. The reaction time was 10 min. The data were obtained from the experiments of [1].



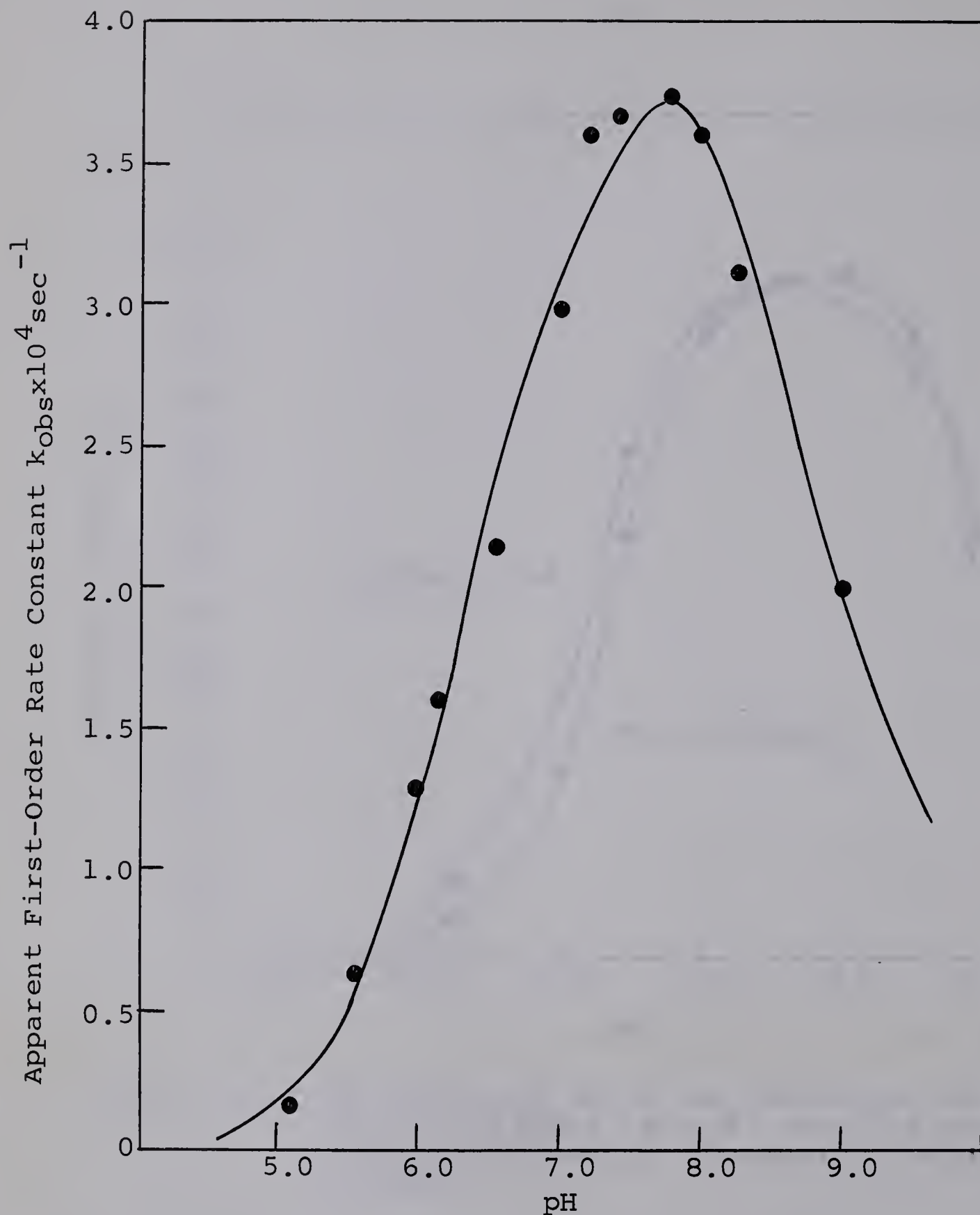
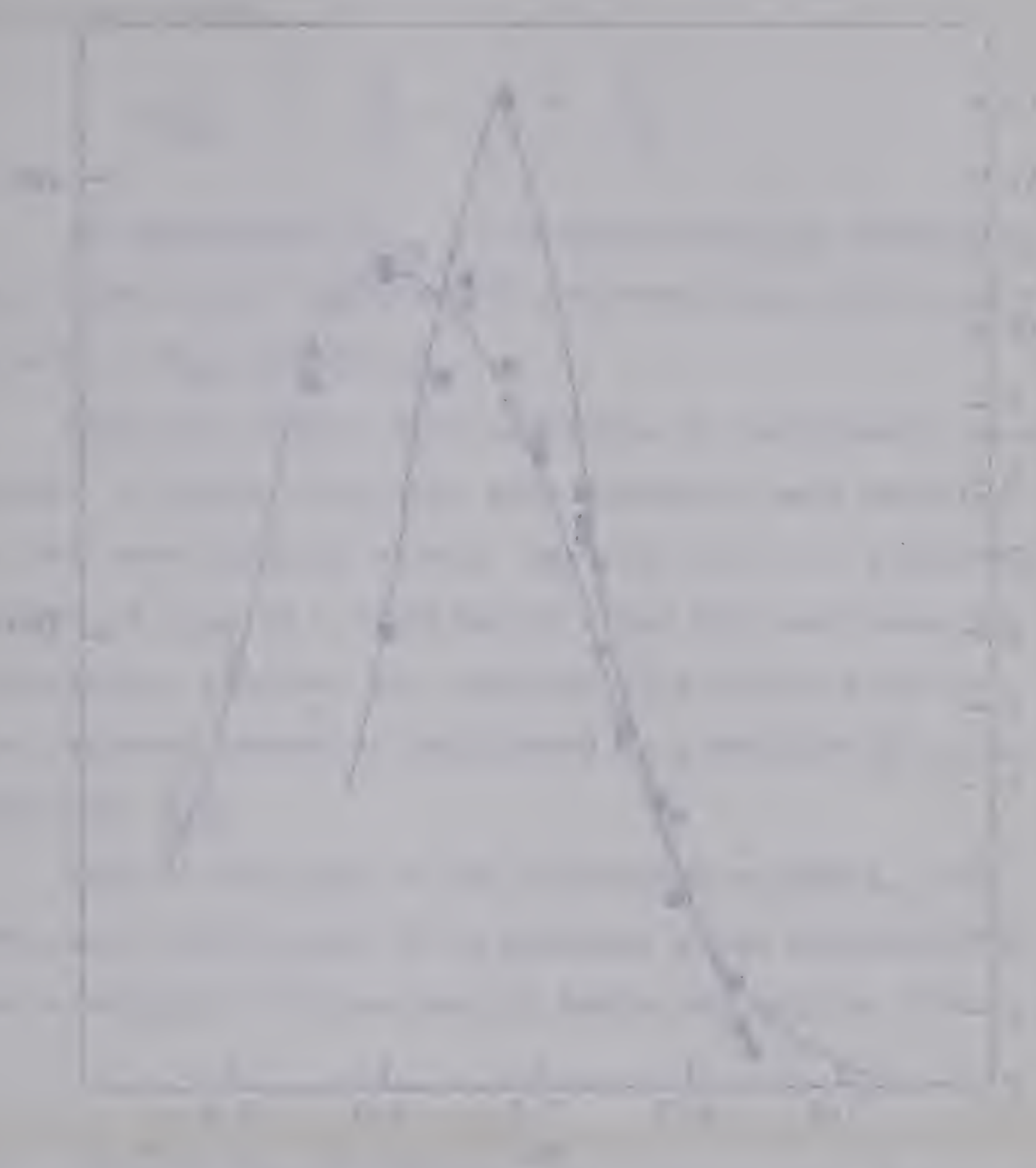


Figure 16. The effect of pH on the rate of inhibition of CHT-B ($1.6 \times 10^{-5} \text{ M}$) with L-TPCK ($1.6 \times 10^{-4} \text{ M}$) at 25° . The ascending arm is a theoretical curve based on the ionization of a group of $pK_a = 6.3$.



The graph shows the height of two objects over time. The solid line represents an object that reaches a maximum height of 10 units at 4 units of time. The dashed line represents an object that reaches a maximum height of 8 units at 2 units of time. Both objects return to the ground at 10 units of time.

The following table provides the data points for the curves shown in the graph. The x-axis represents time in seconds, and the y-axis represents height in feet.

Time (s)	Height (ft) - Solid Line	Height (ft) - Dashed Line
0	0	0
1	4	4
2	8	8
3	10	7
4	10	6
5	8	5
6	6	4
7	4	3
8	2	2
9	1	1
10	0	0

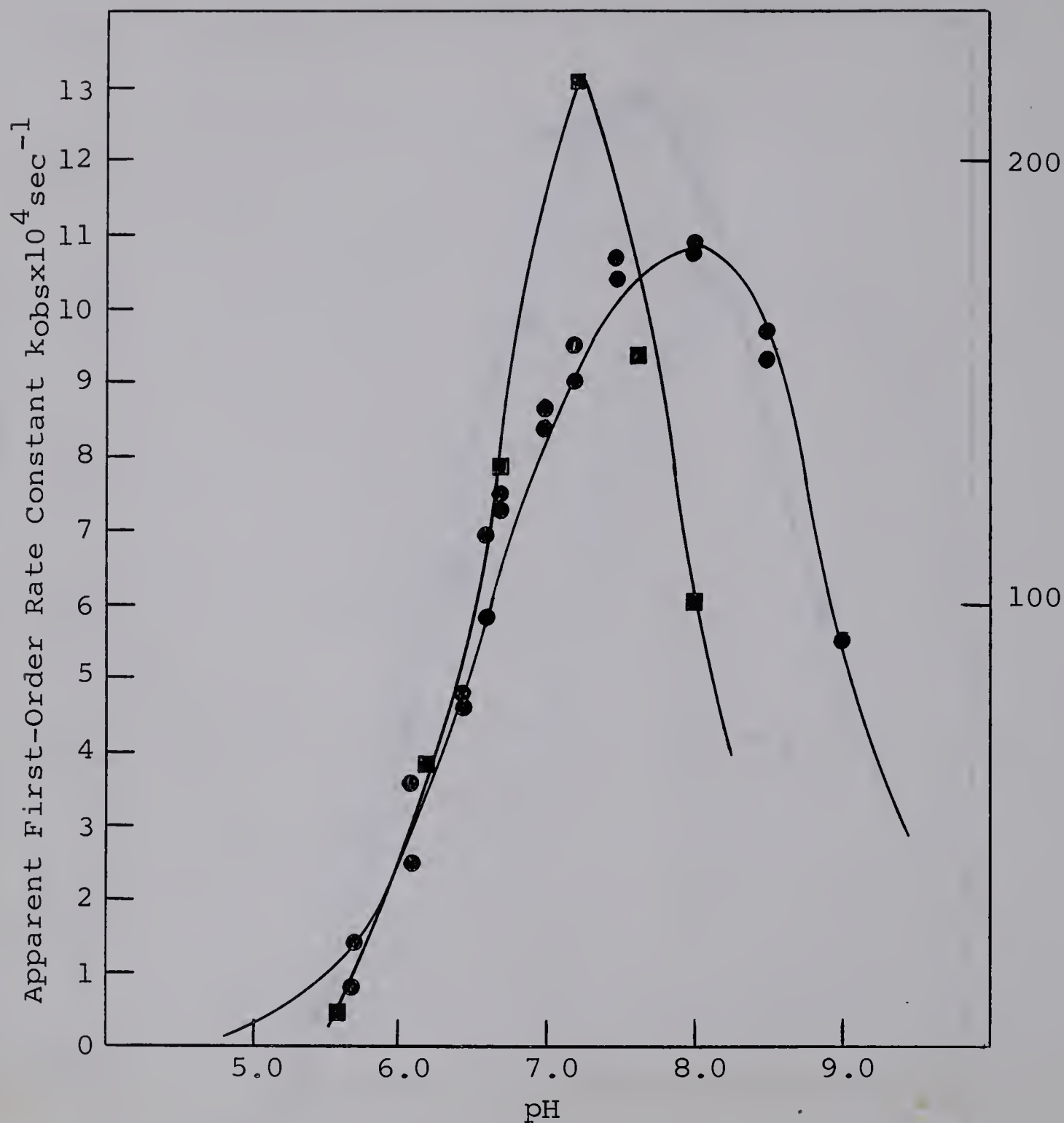


Figure 17. The effect of pH on the rate of inhibition of CHT-A₄ ($1.6 \times 10^{-5} \text{ M}$) with L-TPCK ($1.6 \times 10^{-4} \text{ M}$) at 25°. (●—●). The ascending arm is a theoretical curve based on the ionization of a group of $\text{pK}_a=6.5$. Data of Schoellmann and Shaw (■—■) (104).

Rearranging yields

$$\frac{1}{k_{\text{obs}}} = \frac{K}{k_3} \cdot \frac{1}{I} + \frac{1}{k_3}$$

By determining k_{obs} at various inhibitor concentrations, K and k_3 may be obtained from the slope and intercept of a plot of k_{obs} against $\frac{1}{I}$.

From the limited data available in the present investigation, it appears that the rate constants were obtained under the conditions of case C; that is, $K \sim I$. Since the variation of k_{obs} as a function of I has not been measured in the present studies, the apparent first-order rate constants reported cannot be considered as a measure of k_3 but as equal to $\frac{k_3 I}{K+I}$.

Only in the case of the inhibition of CHT-A₄ with L-TPCK was a calculation of k_3 possible since estimates of K were available from the work of Keith and Smillie (174).

b. Results and Discussion

(i) The Effect of pH on the Rate of Inhibition of CHT-A₄ and CHT-B with L-TPCK

Since L-TPCK predominately alkylates the imidazole group of histidine - 57 in its basic form, it was anticipated that the ascending arm of Figures 16 and 17, CHT-B and CHT-A₄ respectively, might depict the titration curve of the imidazole side chain (105, 106, 108). The curves obtained for CHT-B and CHT-A₄ correspond to the titration curve of an ionizable group of apparent pK_a of 6.3 and 6.5 respectively. The effect of pH on the inhibition of CHT-A₄

and CHT-B with L-TPCK (Figure 15) supports the ionization of a histidine residue. The discrepancies between the data and the theoretical curves in the acidic region may be attributed to various factors. It has been shown (Chapter IV) that L-TPCK is capable of reacting with methionine - 192, three residues removed from the active serine residue. Such a modification would inhibit CHT-A₄ by impeding the proper orientation of substrate in the active site. It is possible that in the acidic region of the curve, where the alkylation of histidine is retarded, the formation of modified methionine could become quite significant. Since CHT-B contains four methionines instead of two present in CHT-A₄, it is conceivable that modification of methionine residues could be enhanced and thus exert a greater influence on the nature of the ascending arm in studies with CHT-B.

The effect of pH on the binding of L-TPCK to the chymotrypsins may contribute to the shape of the ascending arm. Hammond and Gutfreund (96) have observed that the K_m of N-acetyl-L-phenylalanine ethyl ester is invariant between pH 6.5 and 8.0. In addition, Cunningham and Brown (95) have found that the K_m of N-acetyl-L-tryptophan ethyl ester is independent of the pH between 6.1 and 8.0. However, recent spectral studies in this laboratory by Keith and Smillie (174) have indicated that the K_I of L-TPCK varies some 10-fold between pH 5 and 7. In view of the variation in affinity of CHT-A₄ for L-TPCK as a function of pH, the binding of the inhibitor could strongly influence the shape of curve in the weakly acid region.

L-TPCK prepared in this laboratory and used throughout the pH-study with CHT-B was later found to contain an inactive

by-product which was suspected to be 1-L-tosylamido-2-phenyl-ethyl hydroxymethyl ketone (L-TPHK). The presence of the impurity accounts for the depression in the apparent first-order rate constants when compared to values in Table Va. A correction factor (2.18), based on the recovery of chlorine in the elemental analyses (Cl: Cal., 10.08%; found, 4.63%) can be employed to bring the data into line with the rate constants determined with authentic L-TPCK (Sigma Chemical Co.).

The presence of L-TPHK could contribute to the discrepancies between the data and the theoretical curve shown in Figure 16. In addition, variations between the ascending arms of curves representing the effect of pH on the inactivation of CHT-B and CHT-A₄ with L-TPCK may be due to the presence of L-TPHK in the CHT-B studies.

The apparent pK_a of 6.3 and 6.5 controlling the ascending arm in Figures 16 and 17 respectively is in agreement with numerous studies in the literature which suggest the ionization of the imidazole group of histidine. Previous studies conducted on the effect of pH on the inactivation of CHT-A₄ with L-TPCK by Schoellmann and Shaw (104) suggest the dependency of the inactivation on a group with an apparent pK_a \sim 6.6. Cunningham (94) and Cunningham and Brown (95) showed that the hydrolysis of ATEE and ATryEE were dependent on a group with a pK_a of 6.7. Extensive studies from Bender's laboratory (56,87,179) suggested that a residue possessing a pK_a \sim 7, which varies slightly upon acylation and deacylation of the enzyme, is a histidine residue. Furthermore, Bernhard and Tashjian (138) have recently observed that the pK_a of deacylation of indoleacryloyl-chymotrypsin was 7.7 as opposed to 7.1 for cinnamoyl-chymotrypsin (181). Enenkel (129) has ob-

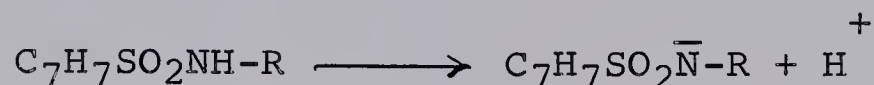
served that a group with an apparent $pK_a \sim 7$ controls the deacylation of cinnamoyl-CHT-B. It is interesting to note that Erlanger et al. (33,182,184) have obtained a curve depicting the effect of pH on the rate of inactivation of CHT-A₄ by DPCC which is remarkably similar to the present studies with CHT-A₄ and L-TPCK. These workers found that the bell-shaped curve was dependent on the ionization of two groups with apparent pK_a 's of 6.6 and 8.7. The fluctuations in the pK_a of the histidine, observed in the preceding studies may result from a perturbation arising from the initial non-covalent binding of the inhibitor or acylating agent to the enzyme.

The pH optimum exhibited in the curves depicting the inhibition of CHT-B and CHT-A₄ with L-TPCK are not identical. CHT-B possesses a broad maximum extending from pH 7.2 to 8.0 which could reflect an inherently lower pK_a for the imidazole group of histidine - 57. Differences in the affinity of CHT-B and CHT-A₄ for L-TPCK could also contribute to the shape of the curve.

Although the data of Schoellmann and Shaw (Figure 17) partially corresponds to the ascending arm of the curve, from the present studies, the pH optimum and the descending arm differ markedly. A pH-optimum between 7.5 and 8.1 appears to be a more realistic value, in the light of numerous kinetic studies reported, than is the value of pH 7.2. In the absence of detailed experimental procedures, one can only assume that the observations of Schoellmann et al. (104) for the effect of pH in the alkaline region, reflects a specific buffer effect not unlike that observed between PMCK and cacodylate buffer (Figure 20).

The descending arms of the bell-shaped curves of CHT-A₄ and CHT-B with L-TPCK are, interestingly, very similar. Both appear

to be dependent on a group with an apparent $pK_a \sim 9$. Three possibilities exist to account for the shape of the pH-curves in the alkaline region: (1) the instability of L-TPCK in the alkaline solution could lead to a decrease in the concentration of L-TPCK. Attack by a hydroxide ion on the chloromethyl moiety could produce the inactive hydroxymethyl ketone (L-TPHK); (2) ionization of the tosylamido moiety



would introduce a negative charge onto the inhibitor which could disrupt the binding of L-TPCK to the enzyme, and (3) the ionization of a group on the enzyme which influences the conformation of the active site could result in a decrease of the enzyme affinity of the reagent.

That the decomposition of L-TPCK in alkaline medium does not account for the reduction in activity has been proven by kinetic studies with L-TPCK. Preincubation of L-TPCK at pH 8.5, followed by an adjustment of the pH to 7.0 and addition of CHT-A₄, yielded the identical apparent first-order rate constant as CHT-A₄ and L-TPCK at pH 7.0 in the absence of the alkaline incubation. A similar result has been recently obtained by Bender (173).

The observed decrease in the inhibition of CHT-A₄ by L-TPCK at alkaline pH was attributed, by Schoellmann and Shaw (104), to the ionization of the tosylamido group ($pK_a \sim 6.9$ in 35% aqueous dimethylformamide). However, Bender (173) has recently obtained a pK_a of 9.9 for the ionization of the tosylamido group. Observations in this laboratory support a pK_a value above 9.5 for the group. Based on the experimental evidence available, it seems likely that the ionization of the tosylamido group does not

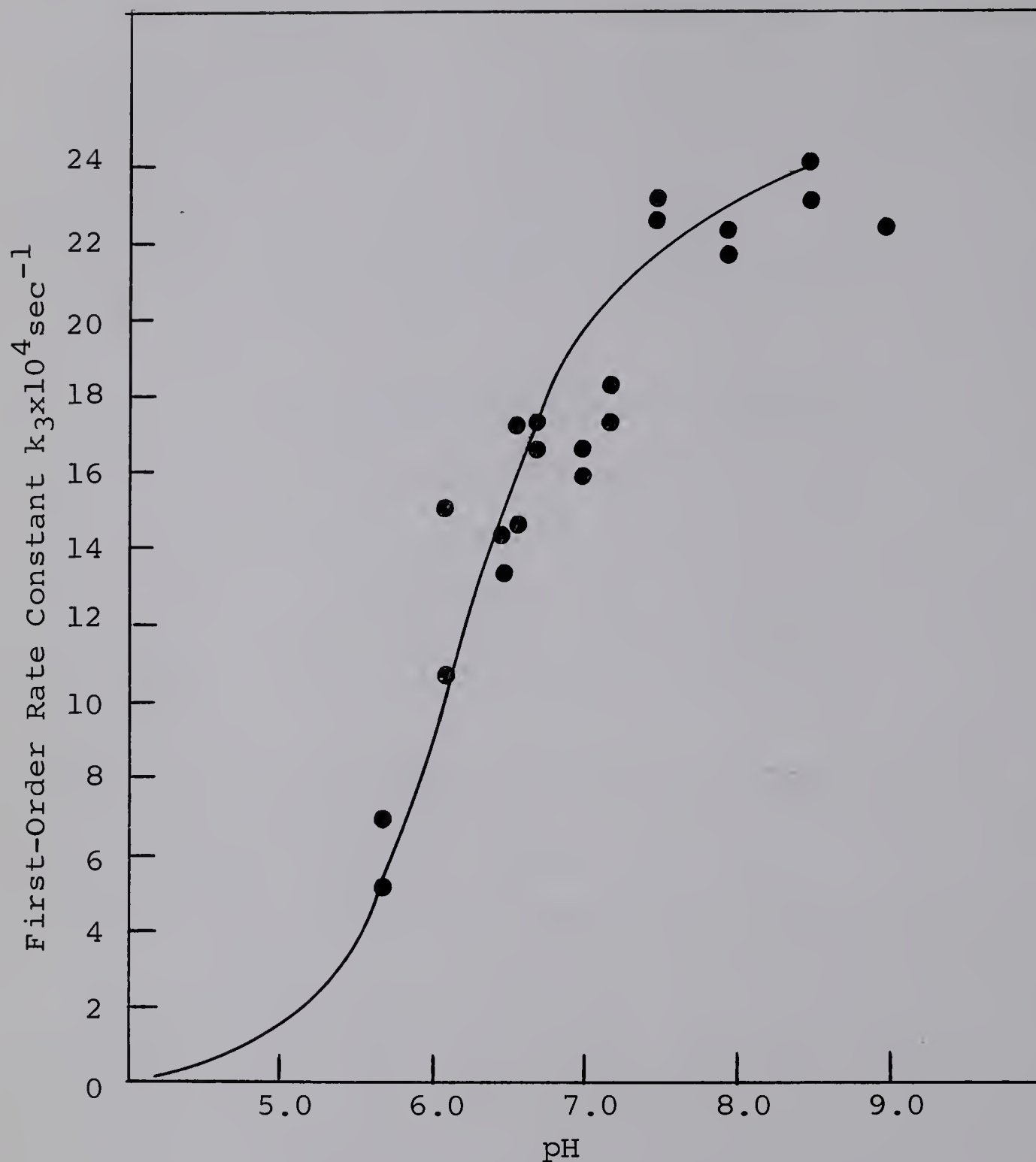


Figure 18. The effect of pH on the rate of inhibition of CHT-A₄ ($1.6 \times 10^{-5} \text{ M}$) with L-TPCK ($1.6 \times 10^{-4} \text{ M}$) at 25° . The first-order rate constant (k_3) calculated from the expression:

$$k_3 = \frac{k_{\text{obs}} (K_m + I)}{I}$$

The theoretical curve presented is based on the ionization of a group $\text{pK}_a=6.2$.

contribute to the observed decrease in the rate of inactivation in alkaline medium.

The possibility of the descending arm of the curve depicting the effect of pH on the inhibition of CHT-A₄ and CHT-B with L-TPCK, being controlled by a group ($pK_a \sim 9$) in the chymotrypsins is particularly attractive. Erlanger et al. (33,182,184) have indicated that the descending arm of the bell-shaped curve, showing the effect of pH on the inactivation of CHT-A₄ with DPCC, was controlled by a group with an apparent pK_a of 8.7. This value is in close agreement with the present studies on L-TPCK. Recently, Himoe and Hess (222) have shown that the decrease in the hydrolysis of N-acetyl-L-tryptophanamide in alkaline medium by CHT-A₄ was due to the effect of pH on the formation of the enzyme-substrate complex and not on the steady-state kinetic parameter- k_{cat} . The pH dependency of K_m (app) implied the involvement of a group with an apparent pK_a of 9. Additional studies cited in the Introduction imply that the group possessing the $pK_a \sim 9$ in CHT-A₄ is the α -amino group of isoleucine-16, the N-terminal of the B chain. The amino acid sequence of CHT-A₄ and CHT-B is almost identical in this region of the two molecules (14). Interestingly, the studies of Hofmann and Scrimger (148) and Hofmann, Gertler and Scrimger (228) reveal that this same group may be functioning in trypsin and in elastase.

A plot of k_3 , the first-order rate constant (calculated from k_{obs}) for the decomposition of the EI complex into products, versus pH appears to resemble the theoretical titration curve of a group with a pK_a of 6.2 (Figure 18). The shift in the pK_a dependency of the ascending arm of the curves in Figures 17 and

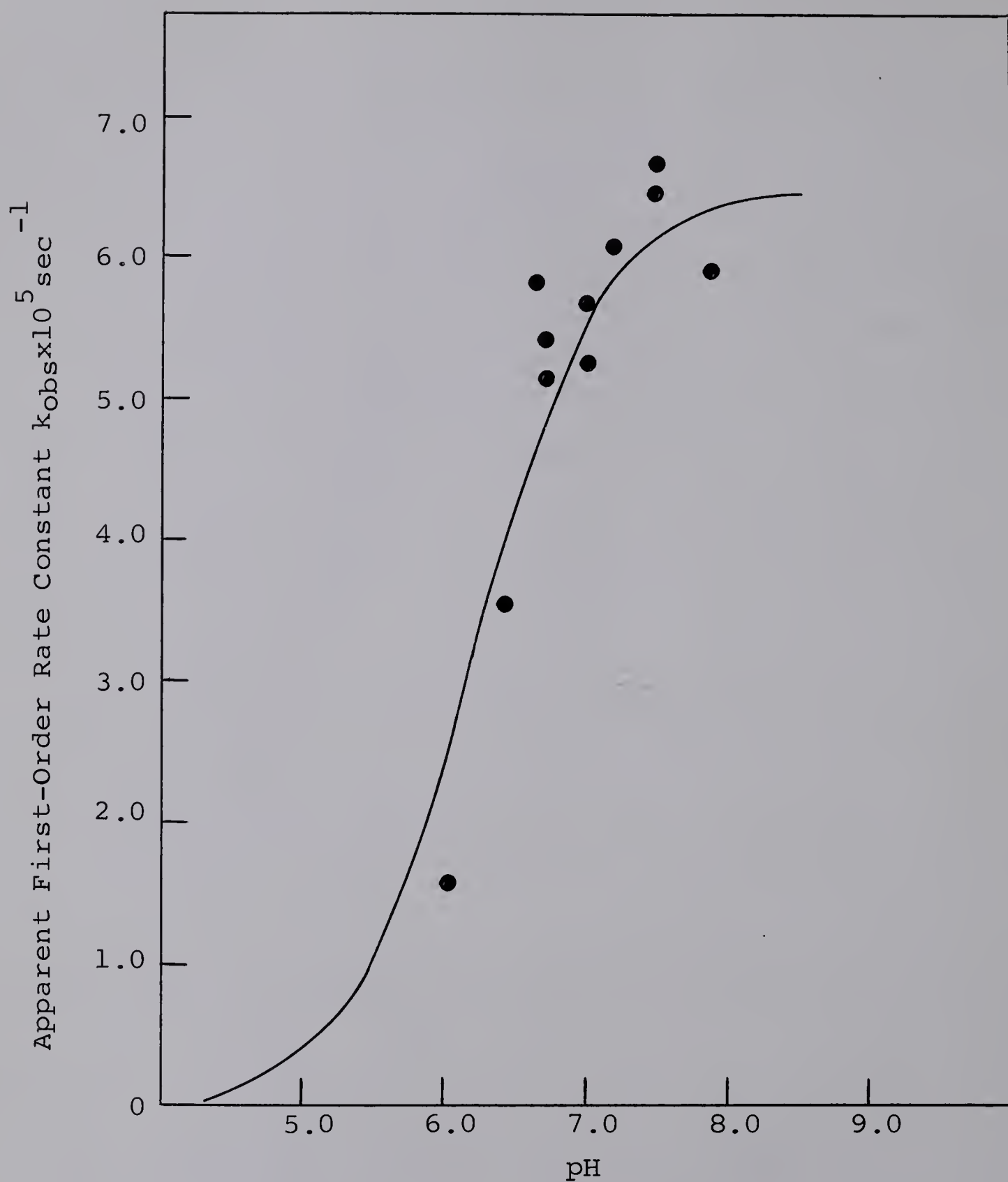


Figure 19. The effect of pH on the rate of inhibition of CHT-A₄ ($1.6 \times 10^{-5} \text{ M}$) and PMCK ($5.64 \times 10^{-3} \text{ M}$) at 25°. The theoretical curve presented is based on the ionization of a group $pK_a=6.3$.

18 indicates the influence of the Michaelis constant (K_I) on the pH inhibition studies.

The reduction in the rate of inhibition (k_{obs}) of CHT-A₄ and CHT-B by L-TPCK in the alkaline region (Figure 17) was not observed when the calculated first-order rate constant (k_3) was plotted against pH as shown in Figure 18. This observation supports the contention that an alteration in the affinity (K_I) of the chymotrypsins for L-TPCK causes the decrease in the rate of inhibition (k_{obs}).

(ii) The Effect of pH on the Rate of Inhibition of CHT-A₄ by PMCK

The effect of pH on the rate of inhibition of CHT-A₄ by PMCK is presented in Figure 19. Since the rate of reaction of CHT-A₄ with PMCK is much slower than with L-TPCK, the apparent first-order rate constants were determined over lengthy intervals, particularly in the acidic region. In the latter region, the limitations of the method began to manifest themselves.

The ascending arms of the bell-shaped curves depicting the effect of pH on the inhibition of CHT-A₄ with PMCK and L-TPCK are indeed very similar. Though the apparent pK_a (~ 6.3 ; imidazole of histidine - 57) for the PMCK-CHT-A₄ system is lower than the pK_a obtained with L-TPCK (~ 6.5), the difference may be actually less since the former value is obtained from a curve drawn through scattered points. Similarities in the curves are notable and suggest that the inhibitors are functioning in much the same manner. The parallelisms observed in the kinetic studies have been extended with structural studies (Chapter IV). The observed rapid decline in the effectiveness of PMCK as an inhibitor as the pH is increased is due, at least in part, to the instability of

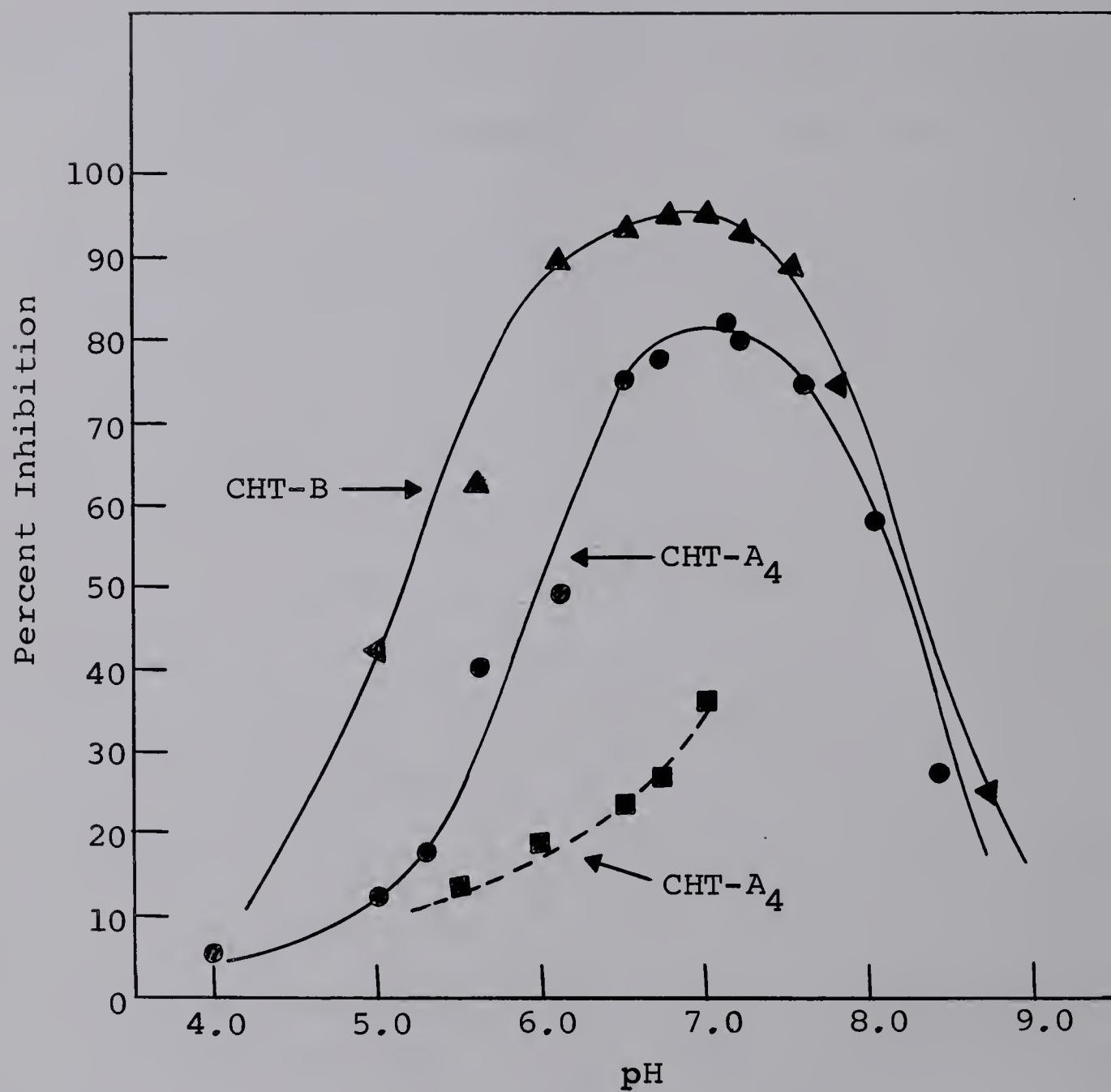


Figure 20. The effect of pH on the inhibition of CHT-A₄ (● — ●) and CHT-B (▲ — ▲) with PMCK. Cacodylate buffer effect on CHT-A₄ inhibition with PMCK (■ — ■).

PMCK in alkaline solution. It has been found that preincubation of PMCK in pH 8 buffer for 24 hours followed by adjustment of the pH to 7 and addition of CHT-A₄, resulted in the recovery of 90% of the enzyme activity 24 hours later.

In contrast to the less rigorous pH studies presented in Figure 20, the effect of pH on the inhibition of CHT-A₄ with PMCK denotes a pH optimum of 7.5 rather than a value of 7.0. This indicates that the rate of inactivation of CHT-A₄ at pH 7.5 is greater than the rate of alkaline destruction of PMCK--a result which was not observed in Figure 20. Certainly the data presented in Figure 19, which is based on apparent first-order rate constants, is the more reliable.

An unexpected observation was the effect of cacodylate buffer (dimethylarsinic acid (CH₃)₂AsOOH) and phosphate buffer on the inhibition of CHT-A₄ with PMCK. The marked depression in the percent inhibition is shown in Figure 20. In order to compare the buffer effects, CHT-A₄ was incubated with PMCK in phosphate, cacodylate and tris buffers at pH 7.0 for 20 hours. The residual activity of the preparations was 50%, 40% and 10% respectively. Phosphate and cacodylate buffers probably exert their effect by reacting with the chloromethyl ketone moiety of PMCK. Interestingly, tris-maleate buffer, which possesses two acidic groups, pKa 1.83 and 6.07, did not noticeably alter the inhibition of CHT-A₄ by PMCK.

The earlier discussions involving the ascending arms of the bell-shaped curves of CHT-A₄ and CHT-B with L-TPCK are applicable to the studies with PMCK. No comment can be made as to the nature of the group, if any, controlling the inhibition of the

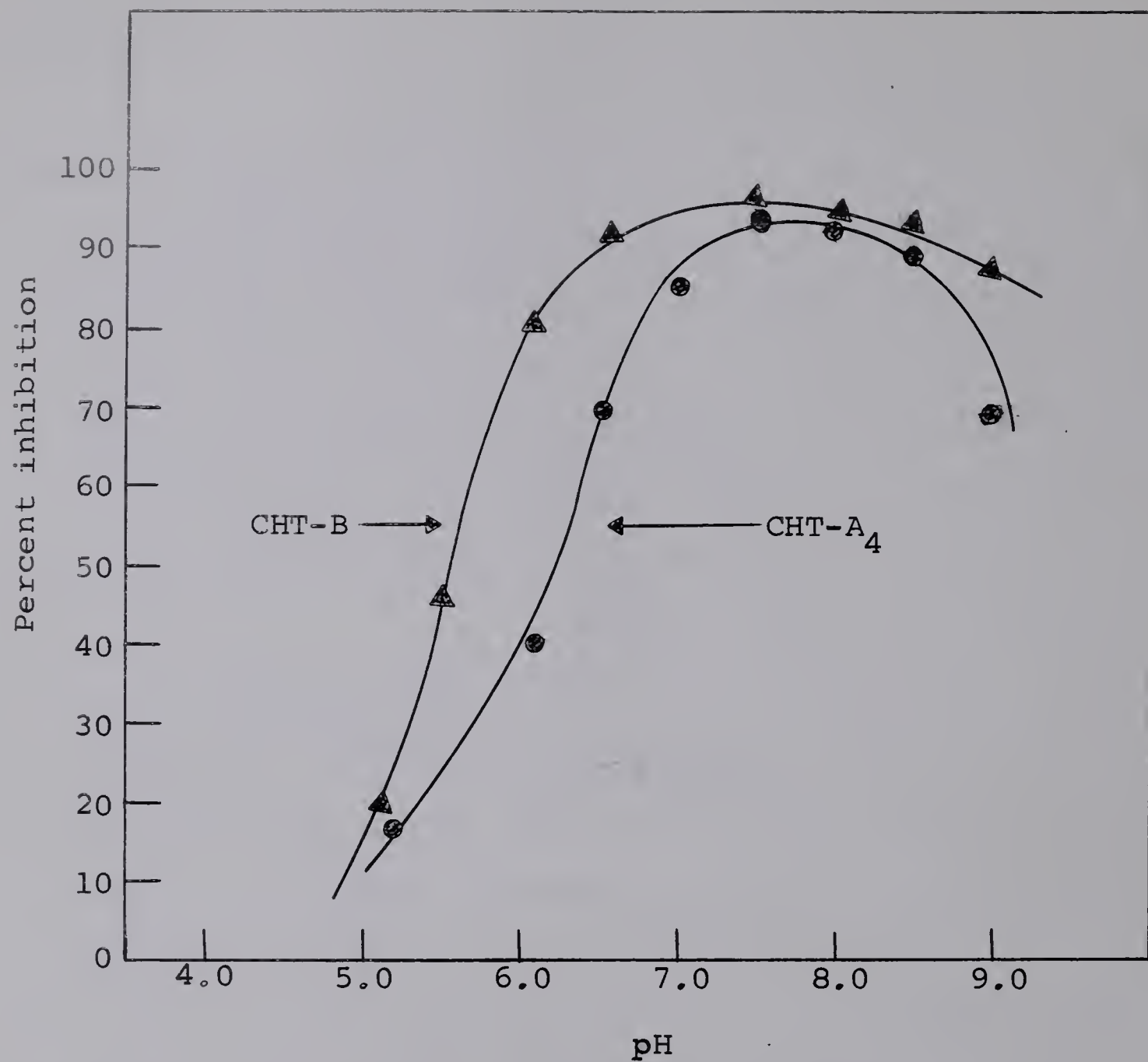


Figure 21. The effect of pH on the inhibition of CHT-A₄ (● — ●) and CHT-B (▲ — ▲) with β SPECK

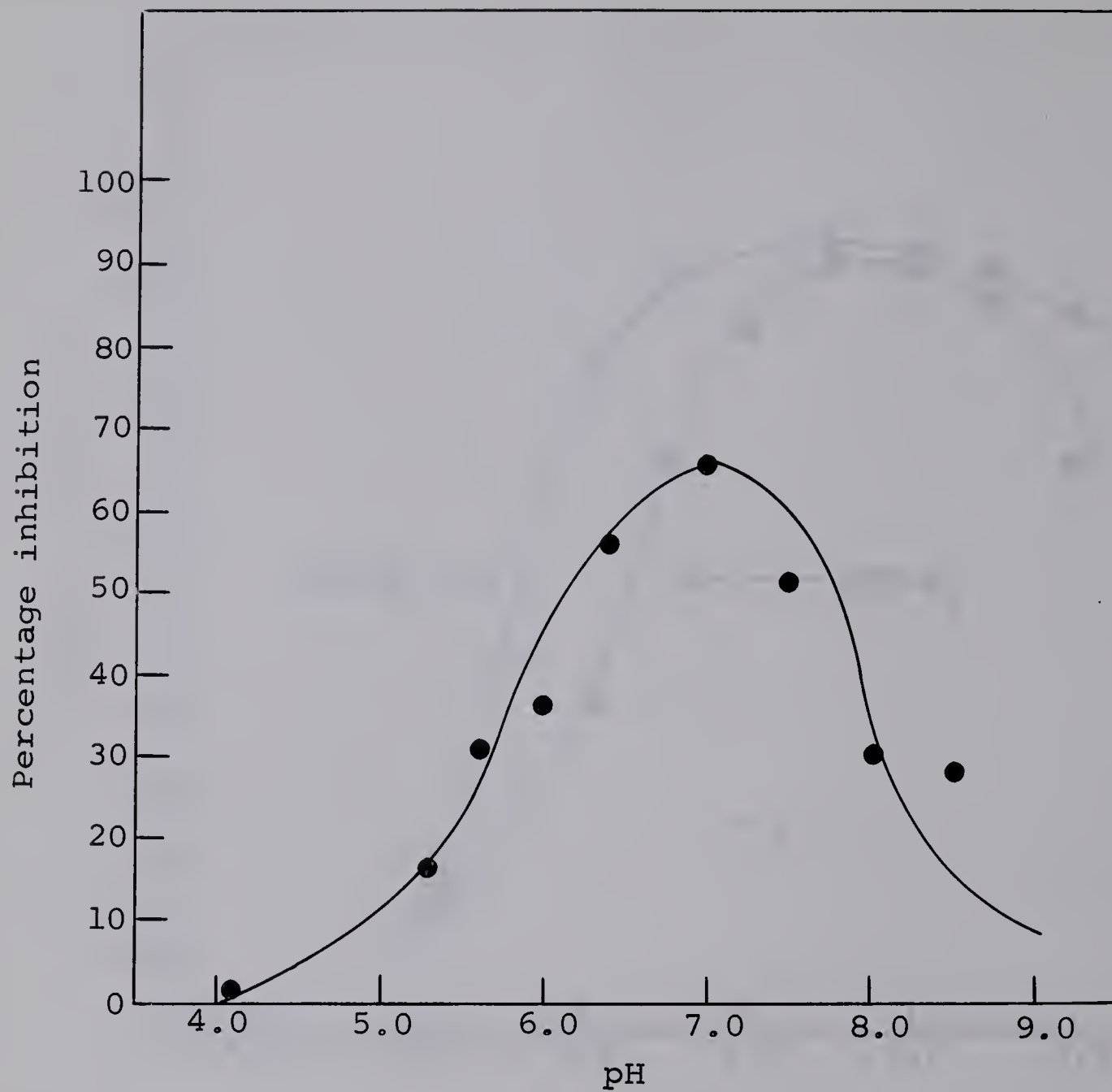


Figure 22. The effect of pH on the inhibition of CHT-A₄ with BCK.

enzyme in the alkaline range since extensive data, corrected for alkaline destruction of PMCK, are not available.

(iii) The Effect of pH on the Inhibition of CHT-A₄ and CHT-B by β PECK

The effect of pH on the inhibition of CHT-A₄ and CHT-B by β PECK is presented in Figure 21. Although the ascending arms of the curves suggest the dependency on the ionization of an imidazole group (CHT-B apparent pK_a \sim 5.6; CHT-A₄ apparent pK_a \sim 6.3), the disparity between the pK_a's is likely not as great as it appears. A definitive study, based on the determination of apparent first-order or second-order rate constants, would be a more reliable indication of differences in the ascending arms of the curves. The present studies suggest that the active sites of CHT-B and CHT-A₄ are not entirely homologous. An inherently lower pK_a for the active histidine - 57 of CHT-B could partially account for the observed curves.

The decrease in the inhibition in the alkaline region may not be dependent on a group with pK_a \sim 9 as was observed with L-TPCK. It is tempting to speculate that the absence of an acyl-amido moiety in β PECK was the cause. However, since the curves were not constructed from the more rigorous apparent first-order rate constant data, such interpretations are rather tenuous.

(iv) The Effect of pH on the Inhibition of CHT-A₄ with BCK

A brief study of the effect of pH on the rate of inhibition of CHT-A₄ with benzyl (phenylmethyl) chloromethyl ketone (BCK) is presented in Figure 22. As witnessed in previous pH studies, the ascending arm of the bell-shaped curve only suggests the ionization of the imidazole group of histidine. Structural studies indicate that BCK reacts with CHT-A₄ in a manner similar to β PECK

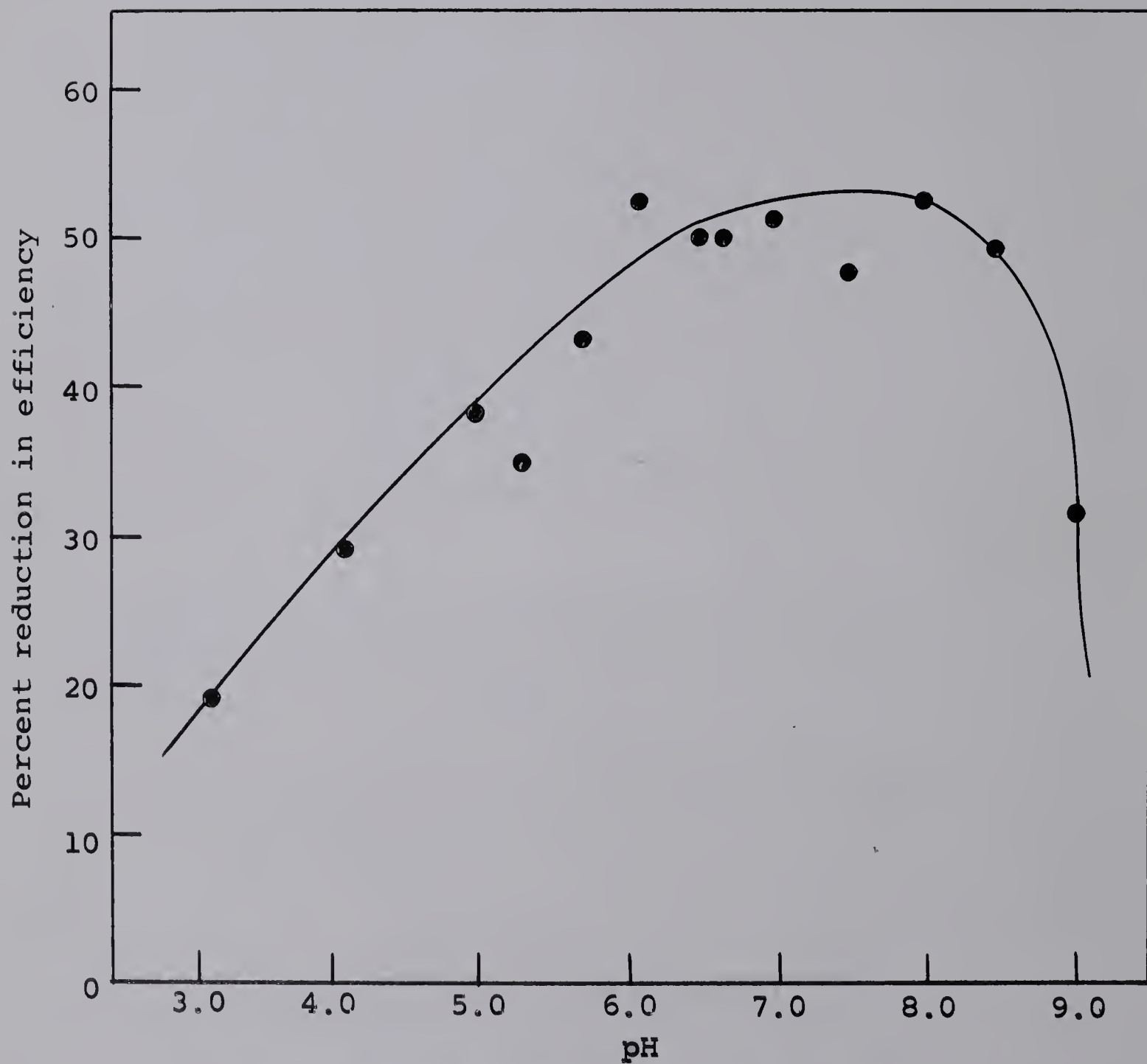


Figure 23. The effect of pH on the inhibition of CHT-A₄ with CA.

except 0.2 residues of histidine rather than 0.4 residues are alkylated, respectively. The instability of BCK in alkaline media appears to be reminiscent of PMCK, with a sharp drop in percent inhibition after pH 7.0.

(v) The Effect of pH on the Inhibition of CHT-A₄ with CA

The effect of pH on the inhibition of CHT-A₄ with CA (C₆H₅.CO.CH₂Cl), shown in Figure 23, cannot be readily interpreted. Structural and chemical studies conducted on CHT-A₄-CA prepared at pH 7.5 revealed that methionine-192 was the sole residue alkylated under these conditions (Chapter IV). That a similar situation exists at lower pH-values is suspected, but experimental support is lacking. The apparent suppression of alkylation from pH 3 to 5 could reflect an alteration in the affinity of CHT-A₄ for CA induced by a discrete conformational change in the vicinity of the active site. Alternatively, the curve could represent the stability of the methionine-sulfonium salt, decomposition being favoured at acidic pH values. It is possible that the "surface" methionine (residue 192) could react readily with CA between pH 3 and 8. The rapid reduction in the percent inhibition in the alkaline region (pH 9) was presumably due to the displacement of the chlorine atom by an hydroxide ion.

5. Apparent First-Order Rate Constants for Bifunctional Reagents with the Chymotrypsins

(a) Methods

A stock solution of CHT-A₄ or CHT-B (1.6×10^{-5} M) was prepared by dissolving 5.0 mg of enzyme in 12.5 ml of 0.05 M Tris-HCl buffer containing 0.05 M CaCl₂, pH 7.2 at 25°. Two aliquots (5.0 ml) were added to test tubes, 250 µl of ethanol was added to each tube, and the solutions were incubated at 25° for five minutes.

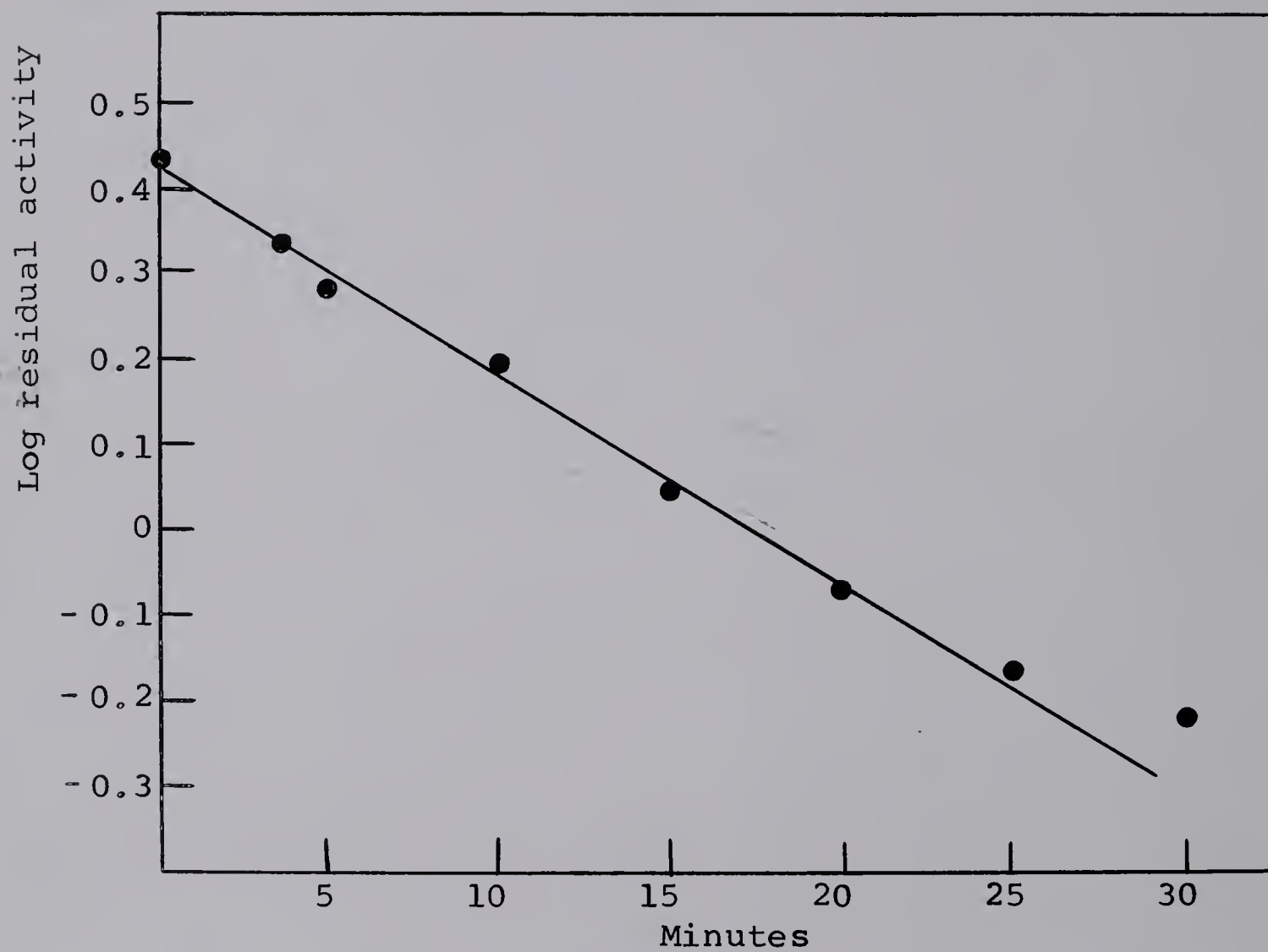


Figure 24a. The inhibition of CHT-A₄ with L-TPCK at pH7.2, 25°.

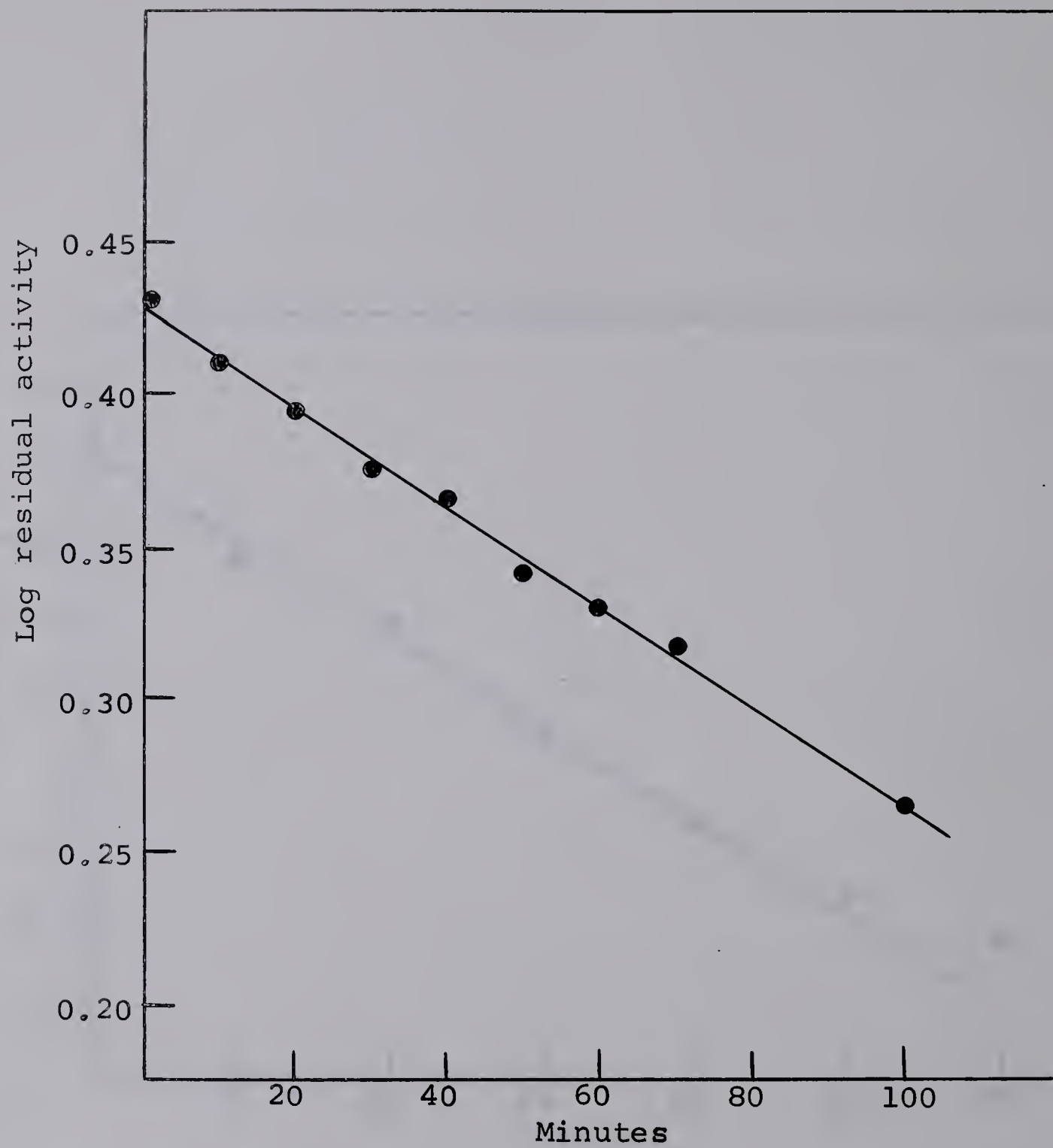


Figure 24b. The inhibition of CHT-A₄ with PMCK at pH7.2, 25°.

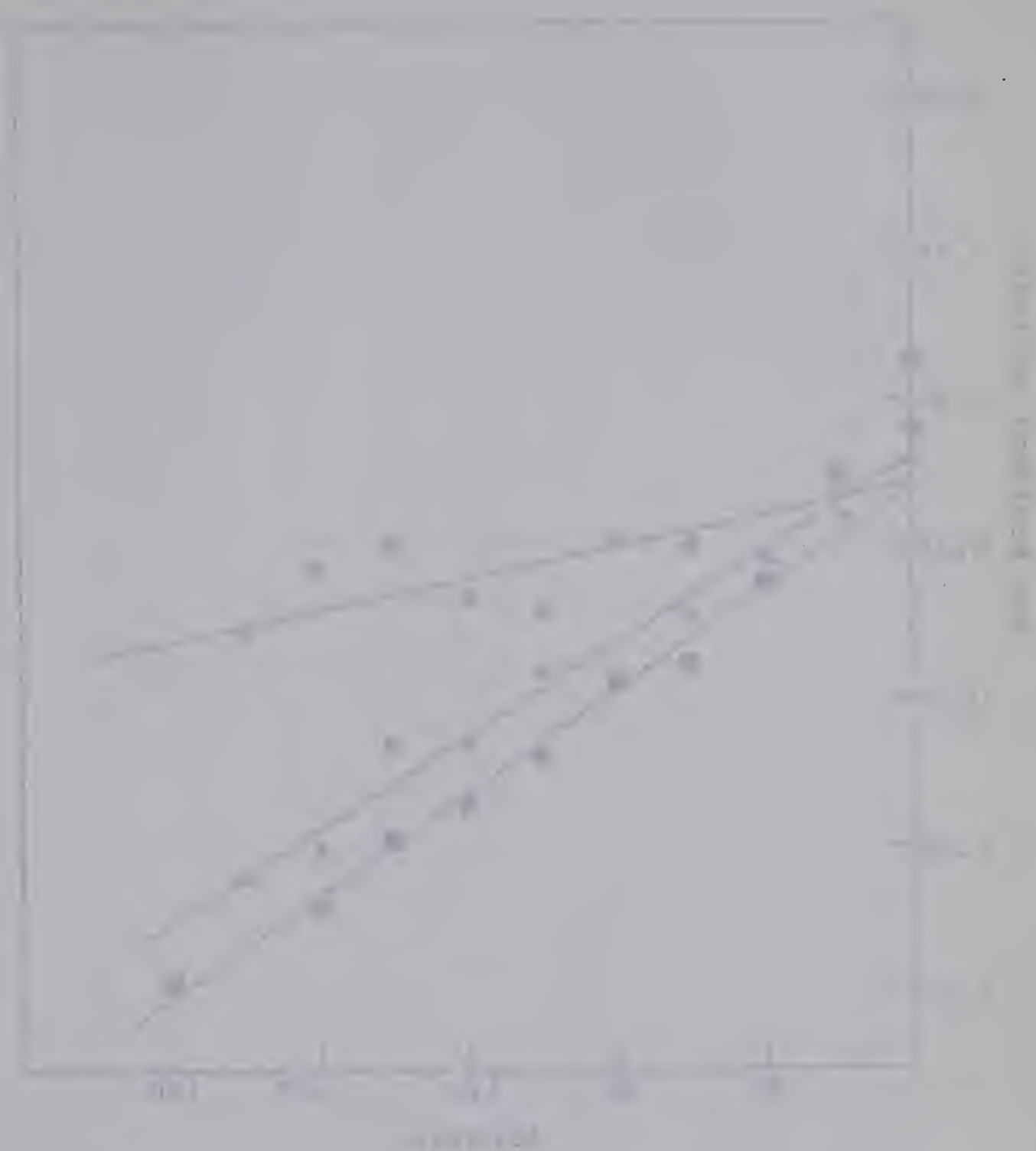


Fig. 1. Dependence of the rate of polymerization on the concentration of the monomer. The reaction was carried out at 30°C. The reaction mixture contained 0.01 mole/l. of the initiator and 0.01 mole/l. of the catalyst.

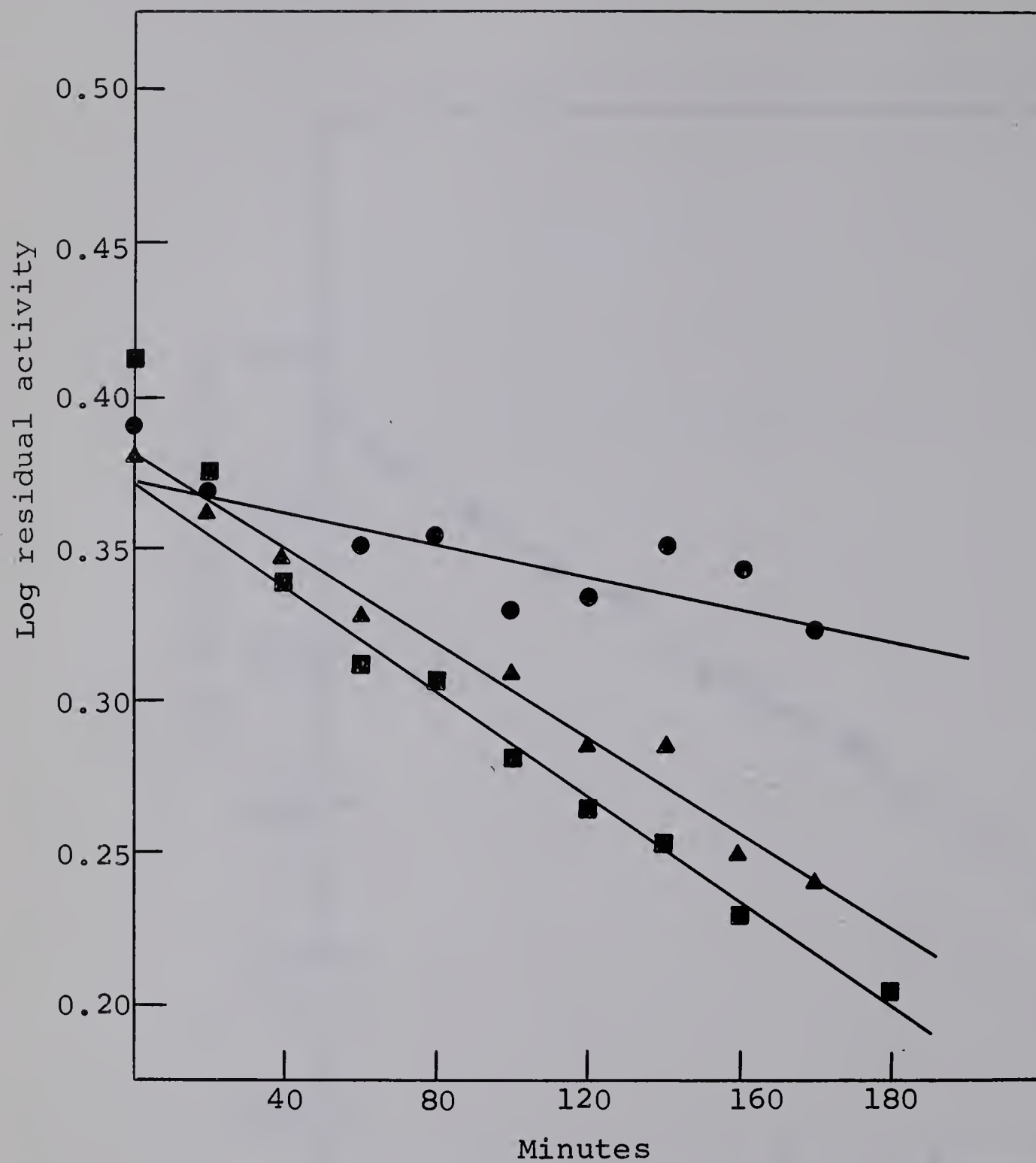


Figure 24c. The inhibition of CHT-A₄ with CA (●—●), BCK (■—■) and βPECK (▲—▲) at pH 7.2, 25°.

TABLE Va

Apparent first-order rate constants of CHT-A₄ and CHT-B with bifunctional reagents

Inhibitor	Structure	CHT-A ₄ $k_{\text{obs}} (\text{sec}^{-1})^{\text{a,b}}$	CHT-B $k_{\text{obs}} (\text{sec}^{-1})^{\text{a,b}}$	Ratio $\frac{\text{CHT-A}_4}{\text{CHT-B}}$
L-TPCK ^c	$\text{C}_6\text{H}_5\cdot\text{CH}_2\cdot\text{CH}(\text{NH}\text{SO}_2\text{C}_7\text{H}_7)\cdot\text{CO}\cdot\text{CH}_2\text{Cl}$	9.50×10^{-4}	3.77×10^{-4}	2.5
PMCK	$\text{C}_6\text{H}_5\cdot\text{O}\cdot\text{CH}_2\cdot\text{CO}\cdot\text{CH}_2\text{Cl}$	6.62×10^{-5}	7.24×10^{-5}	0.8
βPECK	$\text{C}_6\text{H}_5\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{CO}\cdot\text{CH}_2\text{Cl}$	3.00×10^{-5}	5.39×10^{-5}	0.6
BCK	$\text{C}_6\text{H}_5\cdot\text{CH}_2\cdot\text{CO}\cdot\text{CH}_2\text{Cl}$	3.99×10^{-5}	3.34×10^{-5}	1.2
CA	$\text{C}_6\text{H}_5\cdot\text{CO}\cdot\text{CH}_2\text{Cl}$	$\sim 1.1 \times 10^{-5}$	$\sim 1.5 \times 10^{-5}$	0.7

a. 0.05M Tris-HCl containing 0.05M CaCl₂ and 4.8% ethanol, pH 7.2, 25°.

b. Average of two determinations.

c. Purchased from Sigma Chemical Co.

The pH was readjusted to 7.2 and 100 μ l aliquots were removed as blanks and added to 700 μ l of 5×10^{-3} M HCl at 0°. A 50 μ l aliquot of a stock inhibitor solution in ethanol (L-TPCK, 1.65×10^{-2} M; PMCK, 0.58 M; β PECK, 0.17 M; BCK, 0.32 M; CA, 0.33 M) was added at zero time. Aliquots (100 μ l) of the solution were removed at appropriate times and added to dilute HCl as before. Samples were removed from the L-TPCK incubations over a 30 minute period, from the PMCK incubation over a period of 2 hours, and from the β PECK, BCK and CA incubations over a period of 3 hours. Suitable aliquots of the cold HCl solution were assayed against 2.5 ml of 0.01 M ATEE at pH 8.0, 25°. Enzyme concentrations were determined from the absorbance of appropriately diluted stock enzyme solutions. The apparent first-order rate constant was determined from a plot of log residual activity versus minutes.

(b) Results and Discussion

Apparent first-order rate constants for CHT-A₄ and CHT-B with various bifunctional reagents are presented in Table Va. Plots of log residual activity versus minutes for the inhibition of CHT-A₄ with L-TPCK, PMCK and β PECK, BCK, and CA are shown in Figures 24a, 24b and 24c, respectively.

In accord with earlier observations with DFP and DPCC, (13,129) CHT-B was found to be inhibited more slowly than CHT-A with the reagent L-TPCK. This resistance towards inhibition was not observed with PMCK and β PECK. In fact, the enzyme proved to be more susceptible to these reagents than CHT-A₄. Scrutiny of the structural features of the various reagents suggests an explanation for this phenomenon. Diphenylcarbamyyl chloride, DFP and L-TPCK possess groups capable of binding to two independent

sites on the chymotrypsins, namely, the specific binding site and the acylamido binding site accommodating $-R$ and $-NHR^1$ respectively, of the molecule $R.CH_2CH(NHR^1).CO.X$. PMCK ($C_6H_5.O.CH_2.CO.CH_2Cl$) and β PECK ($C_6H_5.CH_2CH_2.CO.CH_2Cl$) are only capable of entering into a productive complex with the specific binding site through the aromatic moieties.* Assuming that the difference in the rate of inhibition of CHT-A₄ and CHT-B is primarily due to an alteration in enzyme affinity for the bifunctional reagents, then either the specific binding site, the acylamido binding site, or both, in CHT-B are not homologous with CHT-A₄. If the specific binding sites in the chymotrypsins are identical, then one would expect PMCK and β PECK to inactivate the enzymes at nearly identical rates. The data indicates that PMCK and β PECK inactivate CHT-B at a rate slightly greater than for CHT-A₄. Although the mode of inhibition of these reagents is quantitatively different for CHT-A₄ (CHT-B has not been investigated), the data suggests that the active sites of CHT-A₄ and CHT-B may not be identical.

An inherently lower pKa of the histidine residue in CHT-B could contribute to the increased rate of inactivation observed with PMCK and β PECK, at pH 7.2, by creating a larger proportion of uncharged histidine residues to react with the chloromethyl ketone group of the reagents. In addition, a more hydrophobic specific binding site would augment the increase in the rate of inactivation. Support for the latter possibility can be gleaned

*Throughout this work a productive complex denotes the absorption of the reagent on the enzyme surface in such a manner that an irreversible alkylation of an active center residue can occur. A non-productive complex might well occur if the aromatic side chain of the reagents was bound to the acylamido site thus placing the reactive chloromethyl ketone moiety some distance away from a catalytically important group.

from the observation that the K_m of ATEE for CHT-B is 40% less than the corresponding value with CHT-A₄ (13,176). Furthermore, the K_I of indole and β -phenylpropionate were found to be less for CHT-B ($4.7 \times 10^{-4} \text{M}$ and $1.7 \times 10^{-2} \text{M}$, respectively) than for CHT-A₄ ($8 \times 10^{-4} \text{M}$ and $2.5 \times 10^{-2} \text{M}$, respectively) (13,186). In the absence of more extensive kinetic studies with CHT-B, these interpretations are suggestive.

Comparison of the structural features of the bifunctional reagents to the rate at which they inhibit CHT-A₄ and CHT-B strongly suggests that the slower rate of inhibition of CHT-B by DPCC, DFP and TPCK is manifested through the acylamido binding site. The observation that DPCC, DFP and L-TPCK react with CHT-B at a rate 5.0-, 9.1- and 2.5-fold slower respectively than with CHT-A₄, indicates that CHT-B may have less affinity for these reagents. Support for this concept is obtained from PMCK and β PECK which are capable of inactivating CHT-B at an enhanced rate when compared to CHT-A₄. This is presumably due to the absence of an acylamido substituent. Although the amino acid sequence of CHT-A₄ (11) and CHT-B (14,177) has been elucidated, the residues comprising the binding sites in these enzymes are not known. Perhaps the acylamido site in CHT-B differs from the analogous site in CHT-A₄ by containing an aspartic or glutamic acid residue in the place of a neutral asparagine or glutamine residue. If the "histidine-loop" of the chymotrypsins influences the acylamido binding site, then the finding that CHT-B contains two negatively charged residues in the "histidine-loop" as opposed to only one in CHT-A₄, is in accord with more hydrophilic environment at the acylamido site. Other regions of the sequence of CHT-B are known to be more negatively

charged than corresponding sequences in CHT-A₄ and could well comprise segments of the acylamido binding site (14).

The ability of L-TPCK ($\text{C}_6\text{H}_5\cdot\text{CH}_2\text{CH}(\text{NHSO}_2\text{C}_7\text{H}_7)\cdot\text{CO}\cdot\text{CH}_2\text{Cl}$) to inactivate the chymotrypsins at a faster rate than PMCK ($\text{C}_6\text{H}_5\cdot\text{O}\cdot\text{CH}_2\cdot\text{COCH}_2\text{Cl}$) undoubtedly resides in the affinity of the enzymes for these reagents. Requirements for the specific binding site are adequately met by the phenylethyl moiety of L-TPCK but the phenoxymethyl moiety of PMCK, containing an electronegative oxygen atom, does not readily facilitate binding of the reagent. The tosylamido group of L-TPCK is able to bind to the acylamido group through a combination of hydrogen and hydrophobic bonds thus imparting additional binding energy for L-TPCK. Although free rotation exists in L-TPCK, it is restricted somewhat by the bulky nature of the tosylamido substituent. Limitation of the degree of free rotation within the molecule reduces the number of configurations in which L-TPCK may exist, thus, apparently, favouring the particular configuration which leads to the alkylation of the histidine-57 residue.

The sluggish nature of the reaction of PMCK with the chymotrypsins appears to be its inability to bind tenaciously. Unrestricted free rotation exists in PMCK thus retarding proper orientation in the active site. It is capable of binding only through one aromatic residue instead of two as is the case for L-TPCK. Since the acylamido binding site is apparently both hydrophobic and hydrophilic (34, 37, 38, 223) it is likely that PMCK could be bound to this site in an unproductive mode. The net effect would be a marked reduction in the rate of reaction as has been observed.

β -phenylethyl chloromethyl ketone ($C_6H_5 \cdot CH_2CH_2 \cdot CO \cdot CH_2Cl$) closely resembles PMCK—a methylene bridge in the former replacing an ether linkage in the latter. Introduction of the methylene bridge may alter the inherent reactivity of the chloromethyl group and, in addition, may lead to a decrease in the enzyme-inhibitor Michaelis constant (K_I). Interpretation of the kinetic data for β PECK is complicated by the fact that the mode of inactivation of CHT-A₄ is not identical to L-TPCK and PMCK (Chapter IV).

The apparent first-order rate constant for α -chloroacetophenone (CA, $C_6H_5 \cdot CO \cdot CH_2Cl$) with the chymotrypsins, as shown in Table V_a, is at best, an approximation. Considerable scattering of the points was obtained when log residual activity was plotted against minutes (Figure 24c). CA has been shown to selectively alkylate methionine-192 of CHT-A₄ to form a sulfonium salt (Chapter IV). Reduction of the chymotryptic activity towards ATEE is attributed to a decrease in substrate affinity (K_m) rather than to an alteration in k_3 . Schramm and Lawson (48) have shown that CHT-A₄, modified through alkylation by α -bromoacetophenone, contained a methionine sulfonium salt residue and was capable of reacting with DFP in a stoichiometric manner. The rate of alkylation of CHT-A₄ by α -bromoacetophenone ($t_{1/2}$ =15 minutes) was considerably faster than the observed rate of alkylation by the α -chloro derivative studied in this laboratory. The differences in the alkylation rates reflect the generally higher reactivity of bromoethyl ketones due to the ability of the bromine atom to polarize the carbon-halogen bond thus facilitating nucleophilic attack by the lone pair of electrons of the sulfur atom of methionine.

Modification of methionine-192 by oxidation (76,88,91) has revealed an alteration in substrate binding as the cause by the observed decrease in enzymatic activity when assayed with a "rate or efficiency" system (ATEE). The enzymes retained full activity when an "all-or-none" assay, using trans-cinnamoylimidazole, was performed and were capable of reacting with DFP.

6. Conclusions

From the kinetic studies presented, several conclusions may be drawn. The pKa of the "active" histidine-57 residue in CHT-B appears to be lower than the corresponding histidine residue in CHT-A₄. This was suggested by the acid shift of the ascending arm of the curves portraying the effect of pH on the inactivation of CHT-B by L-TPCK, PMCK and β PECK when compared to the corresponding curves for CHT-A₄ which were obtained under identical conditions. The observed disparity in the pKa of histidine may be restricted to alkylation or acylation of the enzymes since studies in this laboratory (129) have indicated that deacylation of cinnamoyl-CHT-B and cinnamoyl-CHT-A₄ are dependent on groups possessing identical pKa's.

The ascending arm of the bell-shaped curves depicting the effect of pH on the apparent first-order rate constant of inhibition of CHT-A₄ by L-TPCK and PMCK were essentially identical to a theoretical titration curve. The data suggests the dependency of the inhibition on a group of pKa \sim 6.5 and 6.3, respectively. That the pKa observed is indicative of the ionization of the imidazole group of histidine-57 is supported by amino acid analyses and structural studies (Chapter IV). Similar observation and deduction pertain to the effect of pH on the inactivation of CHT-A₄ by BCK.

Possibly the specific binding site and most likely the acylamido binding site of CHT-B is not homologous with CHT-A₄. The specific binding site of CHT-B may be more hydrophobic in nature than the corresponding site in CHT-A₄. This is inferred from the slightly enhanced rate of inactivation of CHT-B by β PECK and PMCK. The resistance of CHT-B to inactivation by L-TPCK, DFP and DPCC appears to be imposed by the acylamido binding site. It is suggested that this particular region in CHT-B may be more hydrophilic in nature due to the abundance of negatively charged residues present in the enzyme.

Of particular interest was the finding that the descending arm of the curve representing the effect of pH on the apparent first-order rate constant (k_{obs}) of inhibition of CHT-A₄ and CHT-B by L-TPCK was dependent on a group with a $pK_a \sim 9$. The instability of L-TPCK and the ionization of the tosylamido group were rejected as factors contributing to the observed reduction of inhibition in the alkaline region. The inhibition of the chymotrypsins by L-TPCK in this region may be controlled by the ionization of the α -amino group of isoleucine-16, the N-terminal of the B-chain. Recent studies by Himoe and Hess (222) suggest that the ionization of this group imposes discrete conformational alterations in the active center which lead to a decrease in the affinity of the enzyme for its substrate. That a similar influence is operating in the binding of L-TPCK to the chymotrypsins was supported by the observations that the calculated first-order rate constant, k_3 , did not decrease in the alkaline region.

The reagents studied appear to be truly bifunctional in nature. Competitive inhibitors such as β -phenylpropionate are

capable of protecting the chymotrypsins from irreversible inactivation by L-TPCK and PMCK. This infers that the bifunctional reagents are non-covalently bound to the active site of the enzymes prior to covalent attachment through the chloromethyl ketone group.

The kinetic studies presented suggest new avenues of investigation which could enhance our understanding of CHT-B. Determination of the binding constant (K_I) for CHT-A₄ and CHT-B with L-TPCK, PMCK and β PECK, following the techniques utilized by Erlanger et al. (33) and Fahrney and Gold (53), would be expected to reveal a difference in the affinity of the enzymes for these reagents. Such knowledge would reflect the nature of the binding sites (specific and acylamido) in the enzymes.

Although our knowledge of the amino acid sequence and the catalytic residues in the active site of CHT-B is quite extensive, detailed kinetic studies are lacking. The present fragmentary evidence indicates that CHT-B possesses subtle variations in the nature of the active site as compared to CHT-A₄. A more definitive study directed towards a comparison of the various kinetic parameters of CHT-B and CHT-A₄ would possibly yield more substantial evidence for differences in their catalytic activities.

IV. ELUCIDATION OF THE MODE OF INHIBITION OF THE CHYMOTRYPSINS WITH BIFUNCTIONAL REAGENTS.

1. Introduction

The involvement of a histidine residue in the chymotryptic hydrolysis of synthetic substrates has been inferred from kinetic (94, 96), photooxidation (88, 97) and alkylation studies (98). Although these investigations strongly implicated the presence of histidine, unequivocal evidence was not obtained until the selective alkylation of a single histidine residue by L-TPCK was demonstrated by Schoellmann and Shaw (103, 104). This bifunctional reagent was similar to the chymotryptic substrate N-tosyl-L-phenylalanine ethyl ester. The phenylalaninyl side chain and the tosylamido group enabled the reagent to be bound to the enzyme while the chloromethyl ketone group formed a covalent linkage with a residue in the active site. Inhibition of CHT-A₄ by L-TPCK was shown to be stoichiometric in nature and was concomitant with loss of a histidine residue. The particular histidine alkylated by L-TPCK was identified as histidine-57 by Smillie and Hartley (105), Posíšilová et al. (106) and Ong et al. (107, 108).

Physiochemical, kinetic and specificity studies in this laboratory by Enenkel and Smillie (6), Smillie, Enenkel and Kay (20), Enenkel (29) and Parkes (13) have indicated that CHT-B is similar to its isozyme, CHT-A₄. Smillie and Hartley (16, 17) have recently shown that extensive homologies exist

around the disulfide bridges of CHT-B and CHT-A₄. Since CHT-A₄ and CHT-B were inhibited by DFP and DPCC, it was anticipated that L-TPCK would lead to the inactivation of the B enzyme in an analogous manner to that previously demonstrated for CHT-A₄. A study of the mode of inactivation of CHT-B by L-TPCK was subsequently undertaken in an attempt to obtain information on the particular residues involved in the active site.

An investigation into the stereospecificity of the reaction of CHT-A₄ and CHT-B with D-TPCK was prompted by the work of Kallos (44,45) who showed that L-(and D) phenylalaninol ditosylate, similar in structure to TPCK, was capable of inhibiting CHT-A₄ irreversibly. The L-isomer was found to inactivate CHT-A₄ 2.5 times more rapidly than the D-isomer. However, the nature of the inhibition has not been elucidated. In view of this apparent anomaly it was of interest to ascertain the effect of D-TPCK on the chymotrypsins.

The inhibition of CHT-A₄ by 1,2-epoxy-3-phenoxypropane (C₆H₅·O·CH₂·CH^{.O.}·CH₂) (EPOP) was shown by Brown and Hartley (42) to arise from the stoichiometric S-alkylation of methionine-192. Comparison of the structures of L-TPCK and EPOP reveals that the distances between the side chain aromatic group and the reactive moieties are similar yet the chloromethyl ketone of L-TPCK alkylates a histidine residue whereas the epoxide of EPOP forms a sulfonium salt with methionine-192. A molecule comprised of a phenoxymethyl side chain and a chloromethyl ketone group (C₆H₅·O·CH₂·CO·CH₂ Cl, PMCK) was synthesized in an attempt to clarify many of the questions which arose concerning

the role of the various substituents (phenylethyl, phenoxy-methyl, tosylamido, chloromethyl ketone, and epoxide) in directing the alkylation to a histidine or methionine residue. In particular, it was of interest to know whether or not the S-alkylation of methionine was directed by the phenoxymethyl side chain or if it was a result of the stereochemical configuration of the three-membered epoxide ring. Since PMCK was void of an asymmetrical carbon and an acylamido substituent, the role of these moieties could be investigated through a study of this reagent. The replacement of a methylene bridge by an ether linkage in PMCK did not alter the spatial arrangement between the aromatic and chloromethyl ketone groups when compared to L-TPCK. This internal consistency was expected to aid in the elucidation of the role of the various substituents of the inhibitors.

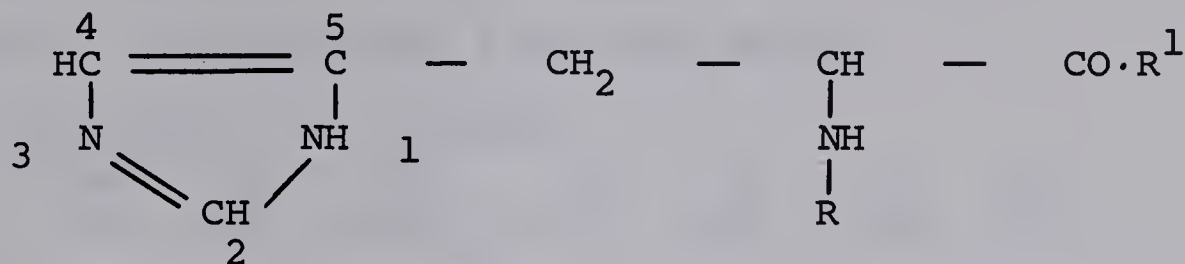
The critical spatial relationship between the aromatic and chloromethyl ketone moiety on the bifunctional reagent which would lead to the alkylation of a histidine residue in CHT-A₄, was examined by employing a homologous series of reagents $C_6H_5 \cdot [CH_2]_n \cdot CO \cdot CH_2Cl$ where $n = 0, 1$ and 2 methylene bridges. These reagents appeared to be capable of alkylating CHT-A₄ since they were either identical or were homologs of the backbone structure of L-TPCK and corresponded closely to the chymotryptic competitive inhibitor β -phenylpropionate ($C_6H_5 \cdot CH_2CH_2 \cdot COOH$). The aromatic group of the bifunctional reagents would be predicted to bind in a common hydrophobic region thereby replacing the reactive chloromethyl ketone moiety at various positions in

the active site. Upon elucidation of the mode of inactivation, if any, through a combination of chemical and structural analyses, it was expected that the structural requirements of a bifunctional reagent for the alkylation of a histidine residue might become apparent.

Similar studies conducted by Schramm and Lawson (48) on a series of phenylalkylamido bromomethyl ketones, $C_6H_5 \cdot [CH_2]_n \cdot NH \cdot CO \cdot CH_2Br$, where $n = 0, 1$ and 2 methylene bridges, revealed that methionine was the only residue alkylated in the modified CHT- A_4 . Comparison of the two homologous series revealed that similar spatial arrangements existed between the aromatic and halomethyl ketones for two members from each series, for example, β -phenylethyl chloromethyl ketone, (β PECK, $C_6H_5 \cdot CH_2CH_2 \cdot CO \cdot CH_2Cl$) and benzylamido bromomethyl ketone ($C_6H_5 \cdot CH_2 \cdot NH \cdot CO \cdot CH_2Br$). Since a methylene bridge does not possess the hydrogen bonding and electronegative properties of an $-NH$ -group, it was of interest to compare the mode of inactivation of the phenylalkyl series with the work reported by Schramm and Lawson (48).

The definitive studies of Gundlach, Stein and Moore (229) and Crestfield, Stein and Moore (230) on the inactivation of ribonuclease by iodoacetic acid and the work of Heinrickson, Stein, Crestfield and Moore (231) and Heinrickson (232) on the inactivation of ribonuclease with haloacids of varying structures, indicated that alkylation of the nitrogen-1 position of histidine-119 or the alkylation of the nitrogen-3 position of histidine-12 were mutually exclusive and were concomitant with the loss of biological activity. Although the accessible nitrogen-3

position, rather than the sterically hindered nitrogen 1 position, was presumed to be the site of alkylation of L-TPCK on histidine-57 of CHT-A₄, experimental evidence was lacking.



The solution to this problem was one of the objectives of the present investigations.

Elucidation of the mode of inhibition of the chymotrypsins by the various bifunctional reagents was conducted on enzyme preparations possessing a minimum of residual activity. The nature of the amino acid alkylated was revealed by amino acid and methionine sulfone analyses after due consideration of those amino acids which are inherently labile or resistant to acid hydrolysis.

Preliminary attempts to elucidate the site of alkylation of L-TPCK in CHT-B by comparing pronase digests of the native and inhibited enzyme were not too encouraging. During this period of the study, Dr. B.S. Hartley at the Laboratory of Molecular Biology, Cambridge, England developed an elegant diagonal paper ionophoretic technique for the isolation and characterization of cysteic acid peptides. Through the application of this technique, pairs of cysteic acid peptides arising from the original half-cystine residues could be readily isolated from performic acid oxidized peptic digests of CHT-A₄. Of considerable interest was the finding by Brown and Hartley (93) that both histidine residues, both methionine residues and

the "active serine" residue of CHTG-A were easily isolated as cysteic acid peptides. The two histidines in CHTG-A were found to be adjacent to the disulfide bridge 42-58 and were contained in the peptides indicated below:

Histidine - 40 Peptide

40	41	42	43	44	45	46
HIS	- PHE	- CYSO ₃	- GLY	- GLY	- SER	- LEU

Histidine - 57 Peptide

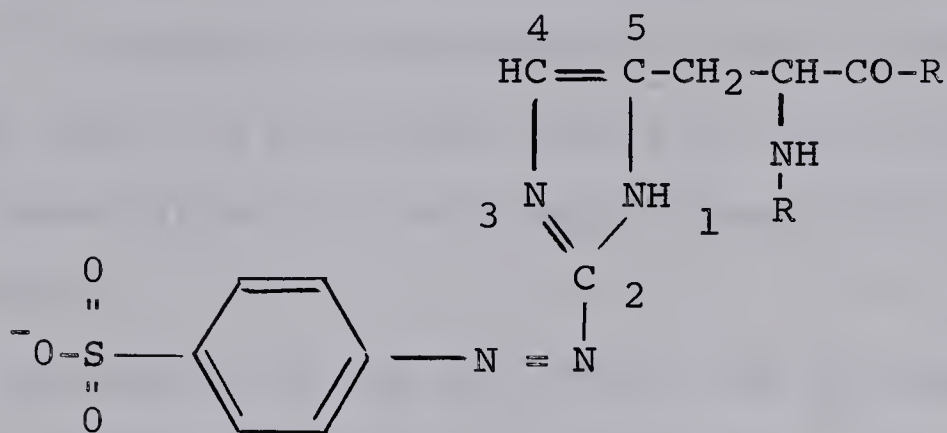
56	56	57	58	59	60	61	62	63	64
ALA	- ALA	- HIS	- CYSO ₃	- GLY	- VAL	- THR	- THR	- SER	- ASP

Identical peptides were isolated from the peptic digests of CHTG-B by Smillie and Hartley (16,17). Using this technique, the site of alkylation of L-TPCK in CHT-A₄ was readily shown to be histidine-57 by Smillie and Hartley (105,189).

Methionine-180 and -192 were present in separate cysteic acid peptides which were located in characteristic positions on the diagonal. S-alkylation of either of these residues could be detected by the alteration in mobility of the particular peptide due to the presence of a positive charge contributed by the sulfonium salt. Identification of methionine -192 as the site of alkylation of CHT-A₄ by EPOP was procured through use of the diagonal technique (42). This technique appeared to be the most convenient and reliable method to study the mode of inactivation of CHT-A₄ and CHT-B with the bifunctional reagents and was utilized extensively in the present study.

Throughout this study, histidine peptides were detected on the paper ionograms by spraying with the Pauly reagent - diazotized sulfanilic acid ($\text{N} \equiv \text{N}^+ - \text{C}_6\text{H}_4 - \text{SO}_3^-$). The chemistry of

this reaction has been reviewed by Hofmann (126). The imidazole side chain of histidine, possessing a free imino group and a hydrogen atom on carbon 2 or 4, will couple with diazotized sulfanilic acid at position C2 or C4 in the presence of dilute sodium carbonate.



REDDISH-ORANGE AZO DYE

The extensive double bond conjugation accounts for the highly colored product. N-alkylation of the imidazole ring prevents coupling of the diazotized aromatic amine, whereas alkylation of carbon 2 or 4 does not hinder the attainment of a Pauly positive reaction since coupling could readily occur at the non-alkylated carbon. A negative Pauly reaction with a modified histidine residue would therefore infer an N-alkylation.

2. Elucidation of the Mode of Inhibition of the Chymotrypsins with L-TPCK and PMCK

(a) Methods

(1) Preparation of Inhibited CHT-A₄ and CHT-B

CHT-A₄ - TPCK and CHT-B - TPCK

CHT-A₄ or CHT-B (200 mgs) was dissolved in 250 mls of 0.1 M tris.HCl buffer, 0.05 M in CaCl₂, pH 7.5. One ml of the solution was removed to serve as a blank. To the solution was added 7.5 ml of ethanol followed by 28 mg of L-TPCK in 5 ml of ethanol to give a molar ratio of L-TPCK/enzyme of 10/1, and

an ethanol concentration of 5%. The solution was stirred at room temperature for 2 1/2 hours and assayed as outlined earlier (Chapter III) against ATEE. Residual activity of the enzymes was found to be about 5%. The solution was brought to pH3.0 with 1M HCl and dialyzed for 24 hours against 3 x 6 litres of 10^{-3} M HCl at 3°. Following the removal of the L-TPCK precipitate by use of a millipore filter (0.8 μ), the solution was freeze-dried to yield approximately 180 mg of inhibited protein.

The preparation of CHT-A₄-D-TPCK, CHT-A₄-N-Methyl-L-TPCK and CHTG-L-TPCK was performed in a similar manner to the procedure outlined above, except that CHT-A₄ or the zymogen was incubated with the reagents (Molar Ratio 10/1) for a 24 hour period. Enzyme assays (ATEE) revealed that the residual activity was 89% in the case of CHT-A₄-D-TPCK and 81% for CHT-A₄-N-Methyl-L-TPCK. The potential activity of CHTG-L-TPCK was not determined. The modified enzymes were freeze-dried and stored at -20° until required.

CHT-A₄ - PMCK and CHT-B - PMCK

The preparation of PMCK inhibited CHT-A₄ and CHT-B was performed by dissolving 200 mg of enzyme in 250 ml of 0.05M tris-maleate buffer, 0.05M CaCl₂, pH7.0. After removal of an aliquot for a blank (1 ml), 7.5 ml of ethanol and 369 mg of PMCK in 5 ml of ethanol were added (molar ratio PMCK/enzyme 250/1). The solution was stirred for 24 hours at room temperature and assayed for residual activity with ATEE. The enzyme solution was brought to pH3.0, dialyzed against 3 x 6 litres

of 10^{-3} M HCl at 3° for 24 hours, and freeze-dried. Approximately 180 mg of the inactivated-enzyme (7% residual activity) was obtained.

Incubation of trypsin (50 mg, Worthington dialyzed and freeze-dried) with PMCK (184.5 mgs molar ratio 500/1) in 50 ml of 0.05M tris-maleate, 0.05M CaCl_2 , at pH7.0 and 5% with respect to ethanol over a period of 24 hours yielded no greater loss of activity than trypsin incubated under the same conditions in the absence of the inhibitor. Trypsin was assayed using benzoyl-L-arginine ethyl ester as described by Enenkel and Smillie (6) .

(ii) Amino Acid Analyses and Methionine Sulfone Analyses

Amino acid analyses of the inhibited chymotrypsins were performed according to the method of Spackman, Stein and Moore (239) on a Beckman 120B automatic amino acid analyzer equipped with the accelerated system. The enzymes (1mg) were hydrolysed with 1 ml of 6N HCl (1:1 dilution of reagent concentrated HCl) in a sealed evacuated tube (15 x 150 mm or 12 x 125 mm Pyrex glass) at $110^{\circ} \pm 2^{\circ}$ for 20 hours. Preparation of the hydrolysate for amino acid analysis was carried out according to the procedure of Moore and Stein (233) . Hydrochloric acid was removed on a Buchler rotary Evapo-mix operating at 50° .

Air oxidation of cysteine was accomplished by dissolving the hydrosylate in 0.5 ml of deionized water and 0.5 ml of 0.2M phosphate buffer at pH6.5. The solution was covered and left to stand at room temperature for 4 hours after which time the pH was adjusted to about 2 by adding 60 μ l of 1M HCl.

The volume was brought to 2.5 ml with 1.5 ml of 0.2N citrate buffer pH2.2. One ml aliquots were analyzed on the 11 cm basic and 56 cm acidic-neutral column of the amino acid analyzer. The number of amino acid residues recovered from the alkylated CHT-A₄ and CHT-B was based on a value of 22.0 residues of alanine for CHT-A₄ (11,188) and CHT-B (20).

Methionine sulfone analyses were conducted according to the method of Moore (235). Performic acid was prepared by adding 1 ml of 30% hydrogen peroxide (Baker Analyzed Reagent) to 9 ml of 90% formic acid (Fisher Certified Reagent). The solution was allowed to stand at room temperature for one hour and was then thoroughly chilled in an ice-bath. One ml of the cold performic acid solution was added to 2.1 mg of native or inhibited CHT-A₄ in a chilled pyrex test tube (15 x 150 mm) and the solution was allowed to stand in an ice-bath for four hours. The oxidation was terminated by adding 0.30 ml of 48% hydrobromic acid (Mallinckrodt Chemical Works). Removal of the acids was accomplished on a Buchler rotary Evapo-Mix operating at 40° under the vacuum supplied by a water pump. The oxidized enzyme was hydrolyzed with 2 ml of 6N HCl in a sealed, evacuated tube for 20 hours at 110° + 2°. The residue remaining after the HCl was removed was dissolved in 1.6 ml of 0.2 M citrate buffer pH2.2. For the analysis of methionine sulfone, a 250 ul aliquot was applied to the acidic-neutral column (55 cm) of the Beckman 120 Automatic Amino Acid Analyzer. Since methionine sulfone (maximum 2.0 residues) emerges at the tailing edge of aspartic acid (maximum 23 residues), the

amount of hydrolysate applied to the column is quite critical. The recovery of cysteic acid reported relative to assumed 22.0 residues of alanine was corrected for 94% yield according to Moore (235).

(iii) Diagonal Paper Ionophoresis

A definitive discussion of the Diagonal paper ionophoresis technique has been recently published by Brown and Hartley (132). The experimental methods to be presented herein are designed to acquaint the reader with the technique.

Native (or inactivated) chymotrypsin A₄ or B (40 mgs) was incubated overnight (16 hours) with 4 mg of pepsin in 10 ml of 5% formic acid at 37°. Since pepsin is relatively non-specific, the digests of the chymotrypsins were essentially free of the insoluble "core" material so commonly associated with trypsin digests. Moreover, the pH of 2 required for peptic hydrolysis stabilizes the disulfide bonds of the cystine peptides formed.

The peptic digest was spotted as a 5 x 40 cm band (1 mg/cm) in the center of a full sheet of Whatman 3MM paper. If Whatman #1 paper was used, the digest was spotted at a concentration of 0.4 mg/cm. The broad band of spotted material was sharpened along the centre line by allowing buffer (pH6.5) to move in slowly from both sides. The rest of the paper was wetted and the sheet was subjected to ionophoresis at pH6.5, 50 volts/cm for one hour. After the paper was dried at 30 - 40°, 3 cm side strips were removed, placed on a glass rack and oxidized in a partially evacuated desiccator for two hours in vapours of performic acid generated from a mixture of 98 - 100% formic

acid (19 ml, British Drug Houses, Reagent Grade) and 30% hydrogen peroxide (1 ml) in the bottom of the desiccator. The oxidized strip was sewn onto the centre of a second sheet of Whatman 3MM (or #1) paper, and was subjected to ionophoresis (pH6.5, 50 volts/cm, one hour) at right angles to the original ionophoresis. Staining the sheet with the cadmium-ninhydrin dip reagent of Heilmann et al. (236) and developing the color at 60° for 20 minutes, yielded the familiar diagonal peptide map. Peptides which were unaltered by performic acid oxidation were found to lie in a line diagonally across the paper. However, peptides (cystine containing) which had acquired a negatively-charged cysteic acid residue, as a result of oxidation, were found as pairs or sets of peptides lying off the diagonal and migrating to the anode. Detection of histidine - containing peptides was accomplished by spraying a separate diagonal with the Pauly reagent (see Appendix B). Purification of cysteic acid peptides in sufficient quantities for amino acid analyses and sequence studies could be obtained by subjecting the parent cystine peptide, present on the initial ionophoresis, to an ionophoresis at pH1.8, 3.5 or 6.5 coupled with performic acid oxidation.

On several occasions it has proven useful to subject the peptic digest of an enzyme to ionophoresis at pH6.5, 50 volts/cm for an extended period of time (up to three hours). The ionophoresis at pH6.5 of the oxidized side strip was also prolonged for 2 or 3 hours. Under these conditions, histidine peptides and peptides in the active serine band were well separated and facilitated interpretation of the diagonal (Figure 35).

TABLE V_bAmino Acid Analyses of CHT-A₄-TPCK and CHT-B-TPCK

Amino Acid	CHT-A ₄ ^a	CHT-A ₄ -TPCK ^b	CHT-B ^c	CHT-B-TPCK
Lysine	14	13.4	10.6	11.1
Histidine	2	1.1	2.0	1.1
Arginine	3	3.0	5.1	4.1
Aspartic Acid	22	21.0	19.5	19.7
Threonine	22	21.7	20.3	21.7
Serine	27	28.4	20.6	20.0
Glutamic Acid	15	14.9	18.5	18.9
Proline	9	9.4	12.7	14.1
Glycine	23	22.1	22.4	23.0
Alanine	22	21.3	22.0	22.0 ^d
Half-Cystine	10	10.2	9.5	9.7
Valine	23	20.9	23.5	24.2
Methionine	2	1.9	3.8	4.0
Isoleucine	10	9.9	8.5	8.3
Leucine	19	18.7	18.6	17.2
Tyrosine	4	4.3	3.2	3.0
Phenylalanine	6	6.1	6.8	7.1

a. Data of Hartley (11) corrected for four amino acids released during activation of CHTG-A.

b. Data of Schoellman and Shaw (104)

c. Data of Smillie, Enenkel and Kay (20)

d. Arbitrarily assumed as 22.0 residues.

Moreover, peptides containing methionine sulfonium salt residues were adequately separated from the corresponding native peptides.

Although pH6.5 - 6.5 diagonals were used throughout the present study, diagonals may be readily obtained using pH1.8 ionophoresis. Under these acidic conditions the peptic digest and the oxidized side strip must be positioned near the anode since all peptides will possess a net positive charge. As an example of the application of pH1.8 - 1.8 diagonals to peptide mapping, the reader is referred to a recent paper by Smillie and Hartley (189).

(b) Chemical and Structural Studies on L-TPCK and PMCK Inhibited Chymotrypsins

(i) Amino Acid Analyses and Methionine Sulfone Analyses

The amino acid analyses of CHT-A₄ - TPCK (104) and CHT-B -TPCK are presented in Table V_b. Comparison of the analyses for native and inhibited enzymes reveals the loss of approximately one histidine residue following the inhibition with L-TPCK. The data presented for CHT-B-TPCK is the result of a single amino acid analysis. The apparent loss of 0.9 residues of arginine is likely due to poor recovery in the particular analysis. Although CHT-B contains one ARG-ILE and two ARG-VAL bonds, it would not appear likely that these bonds would be acid resistant since only peptide bonds in which the carbonyl group is contributed by valine or isoleucine are known to be resistant to acid hydrolysis (204). However, low recoveries of arginine are also indicated in the 20 hour hydrolysis of CHT-B-PMCK shown in Table VIII.

TABLE VI

Methionine Sulfone Analyses of Native CHT-A₄ and CHTG-A and Alkylated Derivatives

Amino Acid	CHT-A ₄ ^a	CHT- ^a L-TPCK	CHT- ^a D-TPCK	CHT- ^a N-Methyl L-TPCK	CHTG-A ^a	CHTG-A ^a L-TPCK	CHTG-A PMCK
Cysteic Acid	10.3	10.1	9.9	9.4	10.3	10.1	9.7
Methionine Sulfone	1.9	1.6	1.6	1.5	1.8	1.6	1.5
Glutamic Acid	14.7	15.3	15.2	15.3	15.0	15.3	14.6
Glycine	22.5	22.7	22.8	22.4	23.0	23.4	22.3
Alanine	22.0 ^b	22.0 ^b	22.0 ^b	22.0 ^b	22.0 ^b	22.0 ^b	22.0

- a. Average of two analyses
- b. Arbitrarily taken as 22.0 residues

TABLE VII

Amino Acid Analysis of CHT-A₄-PMCK

Amino Acid	20 Hour Hydrolysis ^a	70 Hour Hydrolysis ^a	Reported	CHT-A ₄ ^b
Lysine	13.5	13.3	13.4 ^c	14
Histidine	1.0	1.0	1.0 ^c	2
Arginine	2.9	2.8	2.9 ^c	3
Aspartic Acid	21.5	21.4	21.5 ^c	22
Threonine	20.7	18.2	21.8 ^f	22
Serine	24.1	17.5	27.0 ^f	27
Glutamic Acid	14.9	14.8	14.9 ^c	15
Proline	9.0	9.9	9.0 ^d	9
Glycine	22.6	22.4	22.5 ^c	23
Alanine	22.0 ^g	22.0 ^g	22.0 ^g	22
Half-Cystine	9.3	6.7	9.3 ^d	10
Valine	21.1	23.2	23.2 ^e	23
Methionine	2.0	2.0	2.0 ^c	2
Isoleucine	9.2	10.0	10.0 ^e	10
Leucine	18.5	18.5	18.5 ^c	19
Tyrosine	3.8	3.6	3.7 ^c	4
Phenylalanine	5.9	5.9	5.9 ^c	6

a. Average of three analyses

b. Data of Hartley (11) corrected for four amino acids released during activation of CHTG-A.

c. Average of 20 and 70 hour hydrolyses

d. 20 hour value

e. 70 hour value

f. Extrapolated to zero time

g. Arbitrarily taken as 22.0 residues

TABLE VIII

Amino Acid Analysis of CHT-B-PMCK

Amino Acid	20 Hour Hydrolysis ^a	70 Hour Hydrolysis ^a	Reported	CHTG-B ^b
Lysine	10.5	10.6	10.6 ^c	10.6
Histidine	1.0	1.1	1.1 ^c	2.0
Arginine	4.4	4.7	4.6 ^c	5.1
Aspartic Acid	19.2	19.1	19.2 ^c	19.5
Threonine	20.3	19.3	20.8 ^e	20.3
Serine	18.8	16.0	20.0 ^e	20.6
Glutamic Acid	17.6	17.6	17.6 ^c	18.5
Proline	13.1	12.9	13.0 ^c	12.7
Glycine	22.1	21.9	22.0 ^c	22.4
Alanine	22.0 ^f	22.0 ^f	22.0 ^f	22.0
Half-cystine	8.7	8.7	8.7 ^c	9.5
Valine	23.1	24.2	24.2 ^d	23.5
Methionine	3.5	3.8	3.7 ^c	3.8
Isoleucine	8.0	8.5	8.5 ^d	8.5
Leucine	17.8	17.9	17.9 ^c	18.6
Tyrosine	3.0	3.2	3.1 ^c	3.2
Phenylalanine	6.4	6.4	6.4 ^c	6.8

a. Average of three analyses

b. Data of Smillie, Enenkel and Kay (20)

c. Average of 20 and 70 hour hydrolyses

d. 70 hour value

e. Extrapolated to zero time

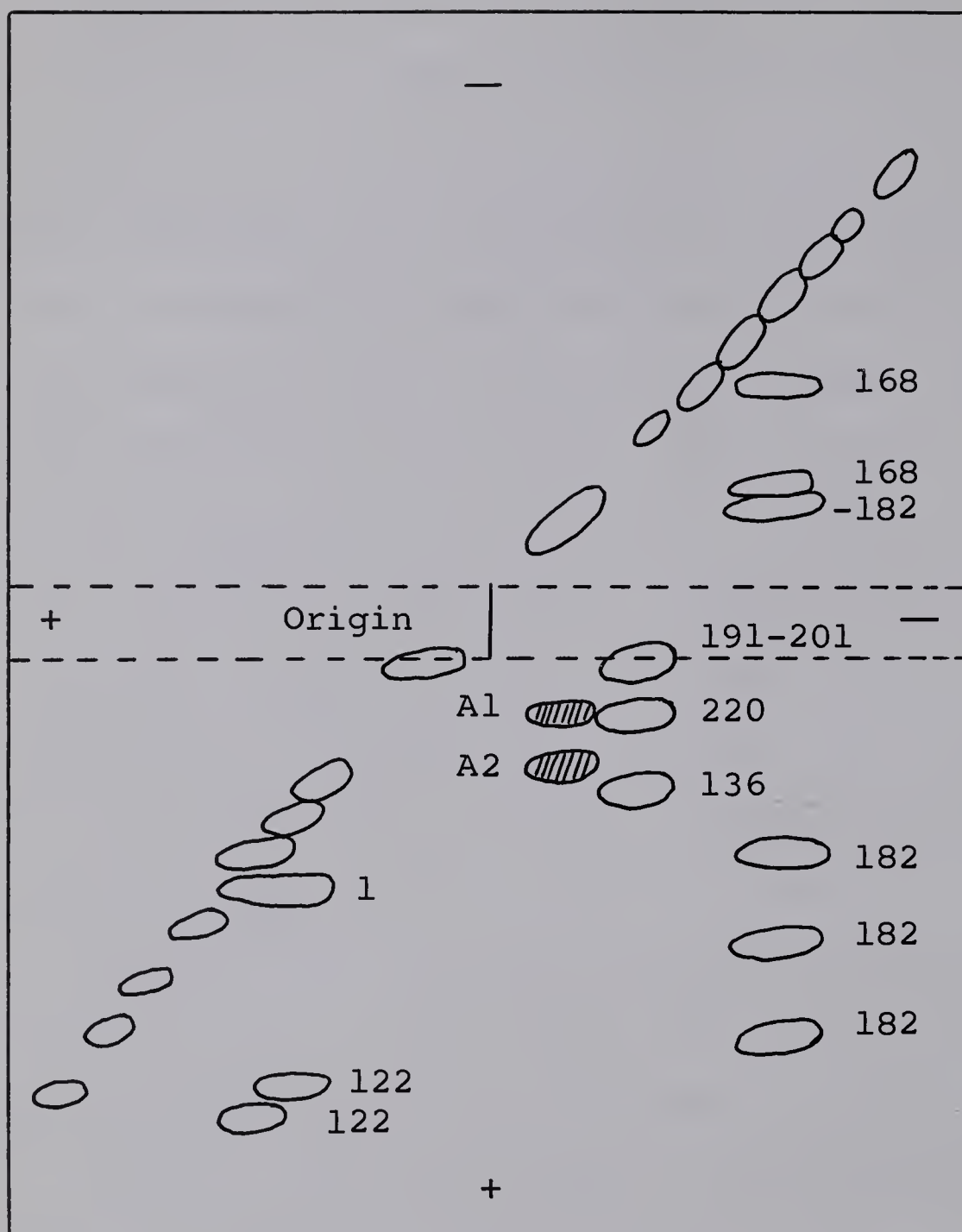
f. Arbitrarily taken as 22.0 residues

Methionine sulfone analyses conducted on native CHT-A₄ and CHT-A₄ modified with L-TPCK, D-TPCK and N-methyl-L-TPCK are reported in Table VI. In addition, analyses of the methionine sulfone content of CHTG-A, CHTG-A-L-TPCK and CHTG-A-PMCK are included.

Based on the studies of Gundlach, Moore and Stein (229), on the stability of methionine carboxymethylsulfonium iodide in performic acid, and on the studies of Moore (235) on the formation of methionine sulfone by performic acid oxidation, the apparent reduction in the recovery of methionine sulfone strongly suggests the presence of methionine sulfonium salts (~ 0.4 residues) in the inhibited CHT-A₄. Interestingly, about 0.3 residues of a sulfonium salt appears to be present in the CHTG-A₄ which has been incubated with L-TPCK and PMCK.

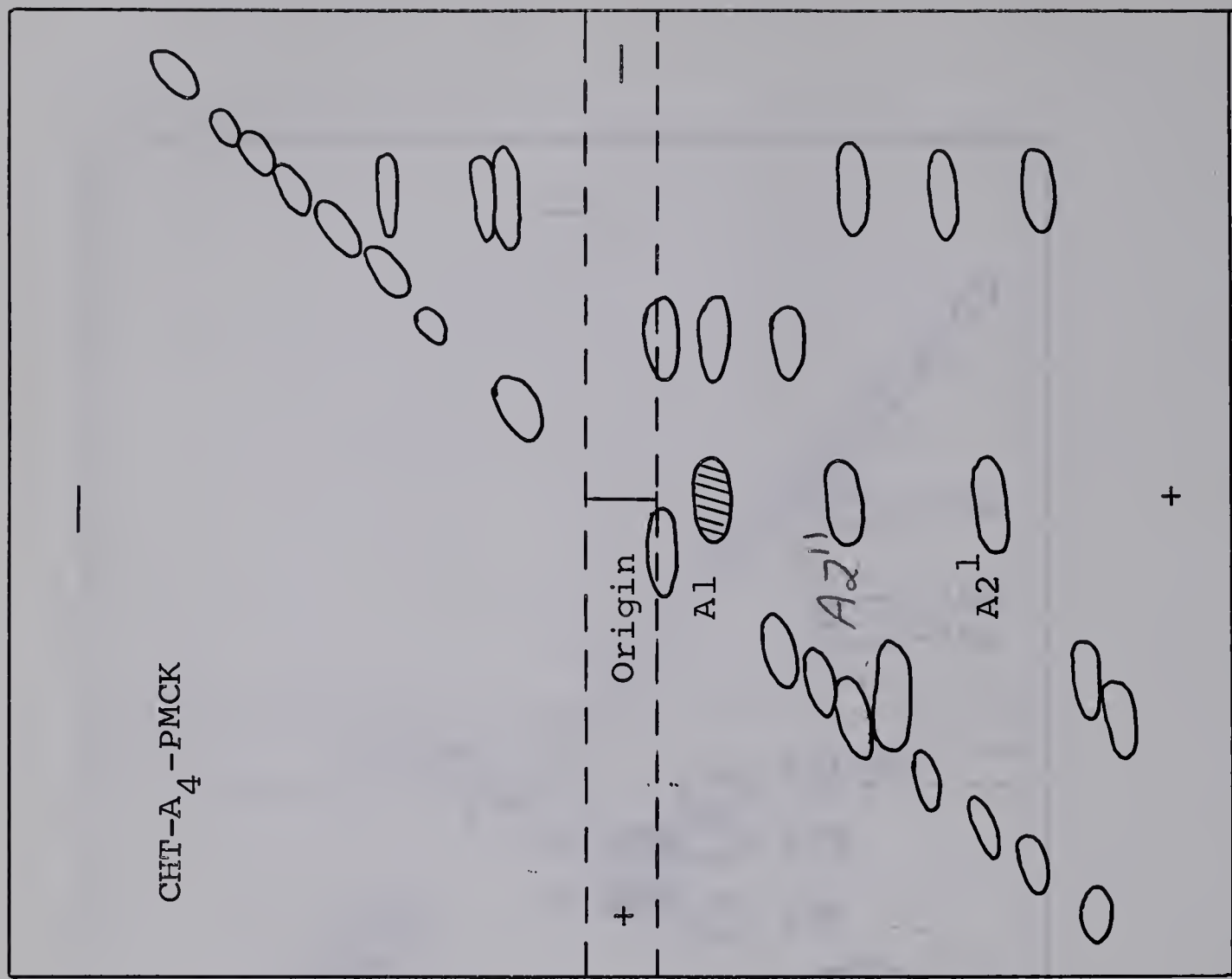
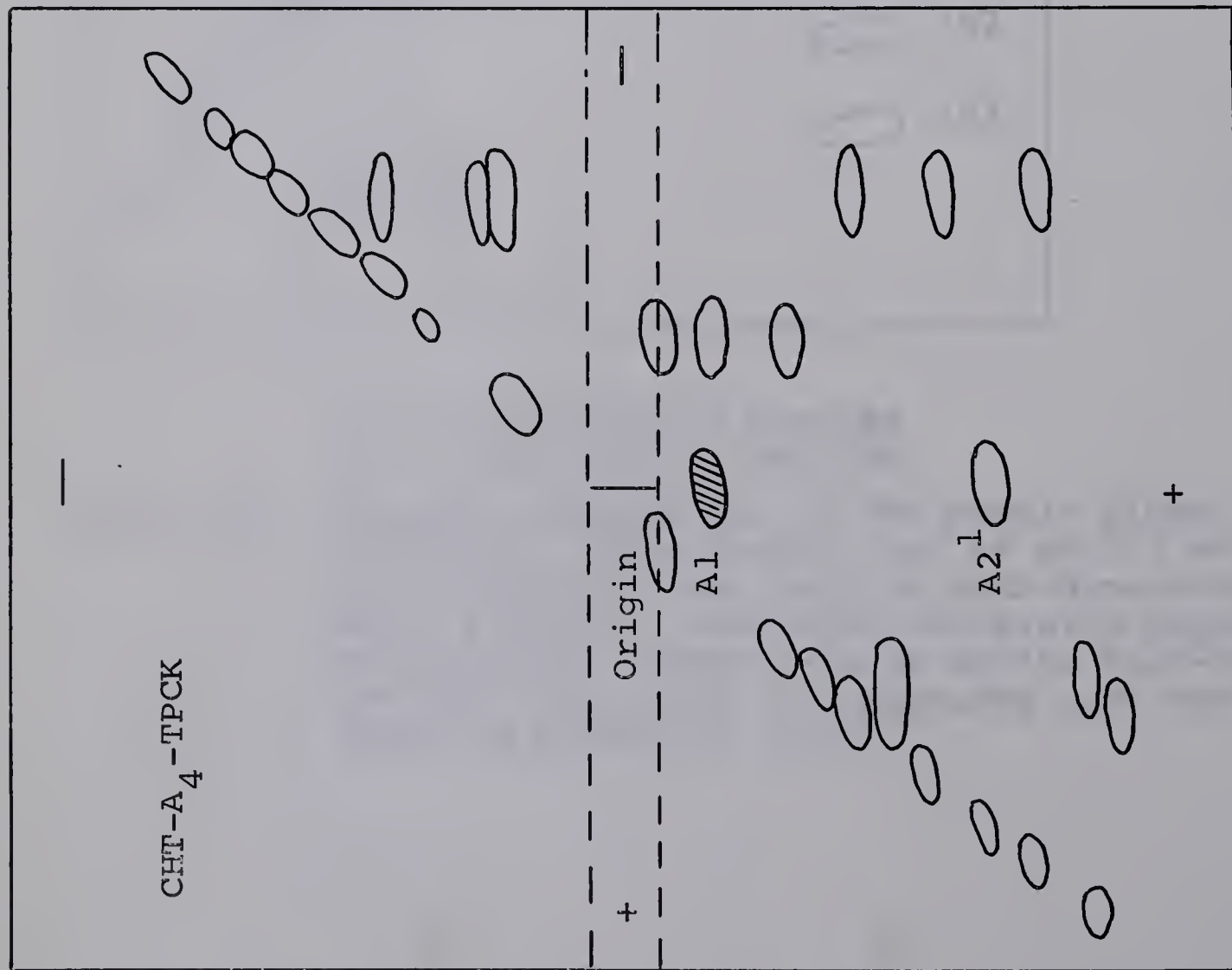
The results of extensive amino acid analyses of CHT-A₄-PMCK and CHT-B-PMCK are presented in Tables VII and VIII respectively. Of significance is the loss of one of the two histidine residues in both PMCK-inhibited chymotrypsins. The reduction of enzyme activity in CHT-A₄ and CHT-B is thus associated with the alkylation of a single histidine residue by PMCK.

Methionine sulfone analyses conducted on CHT-A₄-PMCK indicate that approximately 0.4 residues of methionine sulfonium salt are present. The recovery of selected amino acids, based on alanine as 220 residues (11) was as follows: cysteic acid, 10.3; methionine sulfone, 1.6; glutamic acid, 14.6; and glycine, 22.9 residues. Although methionine sulfone analyses



A1 is histidine-40 peptide
A2 is histidine-57 peptide

Figure 25. Diagonal peptide map of the peptic digest of CHT-A₄. Ionophoresis was at pH 6.5 and 50 volts/cm for one hour in both dimensions. Pauly positive, histidine containing peptides are hatched. Numbers refer to the half-cystine residues from which the peptides were derived (Brown and Hartley (132)).



A1 is histidine-40 peptide

A2 is histidine-57 peptide

A2¹ is alkylated histidine-57 peptide

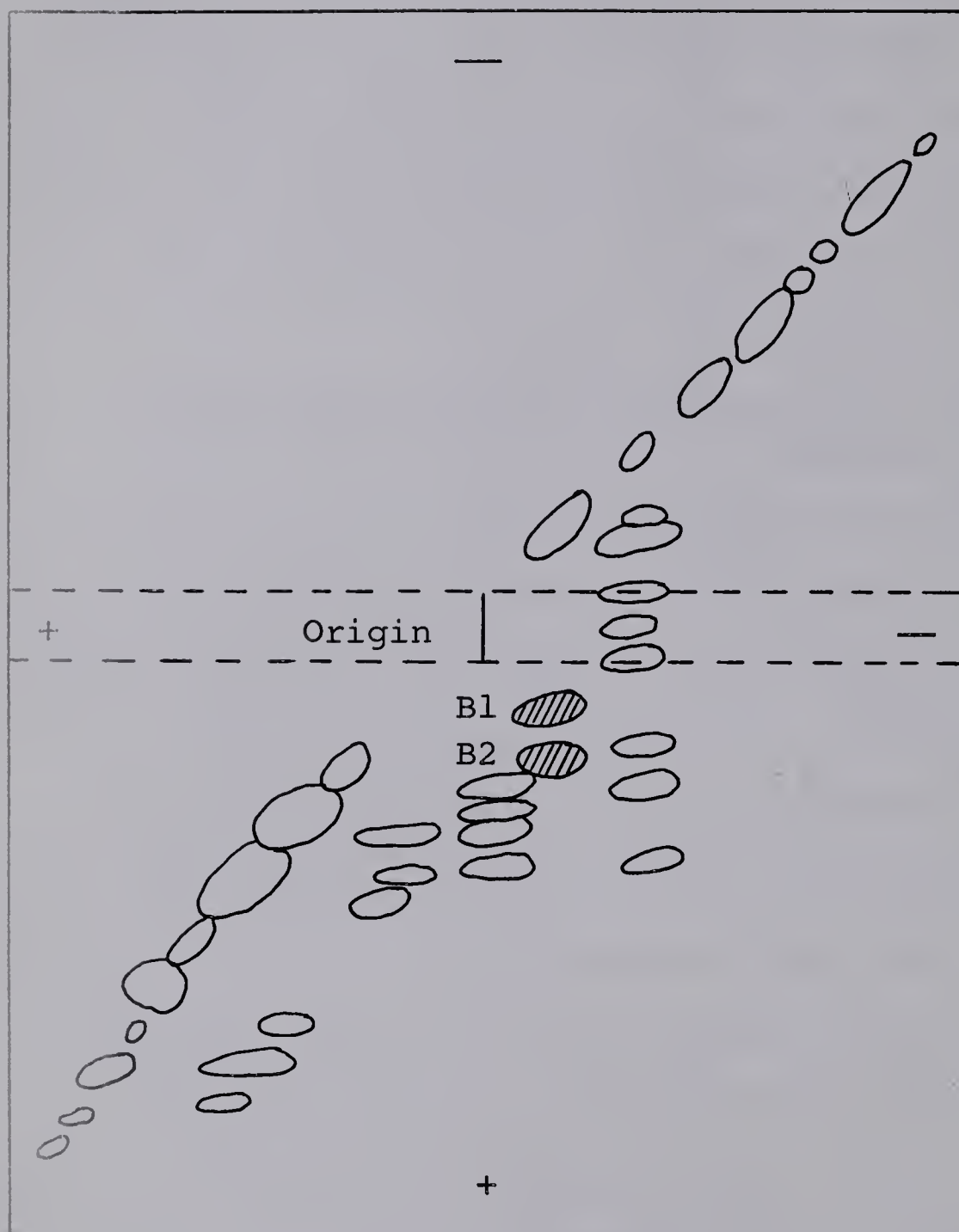
Figure 26. Diagonal peptide maps of the peptic digest of CHT-A₄-TPCK and CHT-A₄-PMCK. Ionophoresis was at pH 6.5 and 50 volts/cm for one hour in both dimensions. Pautly positive, histidine containing peptides are hatched.

were not conducted on CHT-B-PMCK or on CHT-B-TPCK, it appears likely that methionine sulfonium salt residues are present in the alkylated B-enzyme. The increasing recovery of methionine over the 70 hour hydrolysis period (Table VIII) could reflect the regeneration of methionine from a sulfonium salt. Alternatively, however, the presence of an acid resistant VAL-MET sequence (residues 179-180 (14)) in CHT-B could also account for the observed increase.

(ii) Diagonal Peptide Maps of Native and Inhibited Chymotrypsins

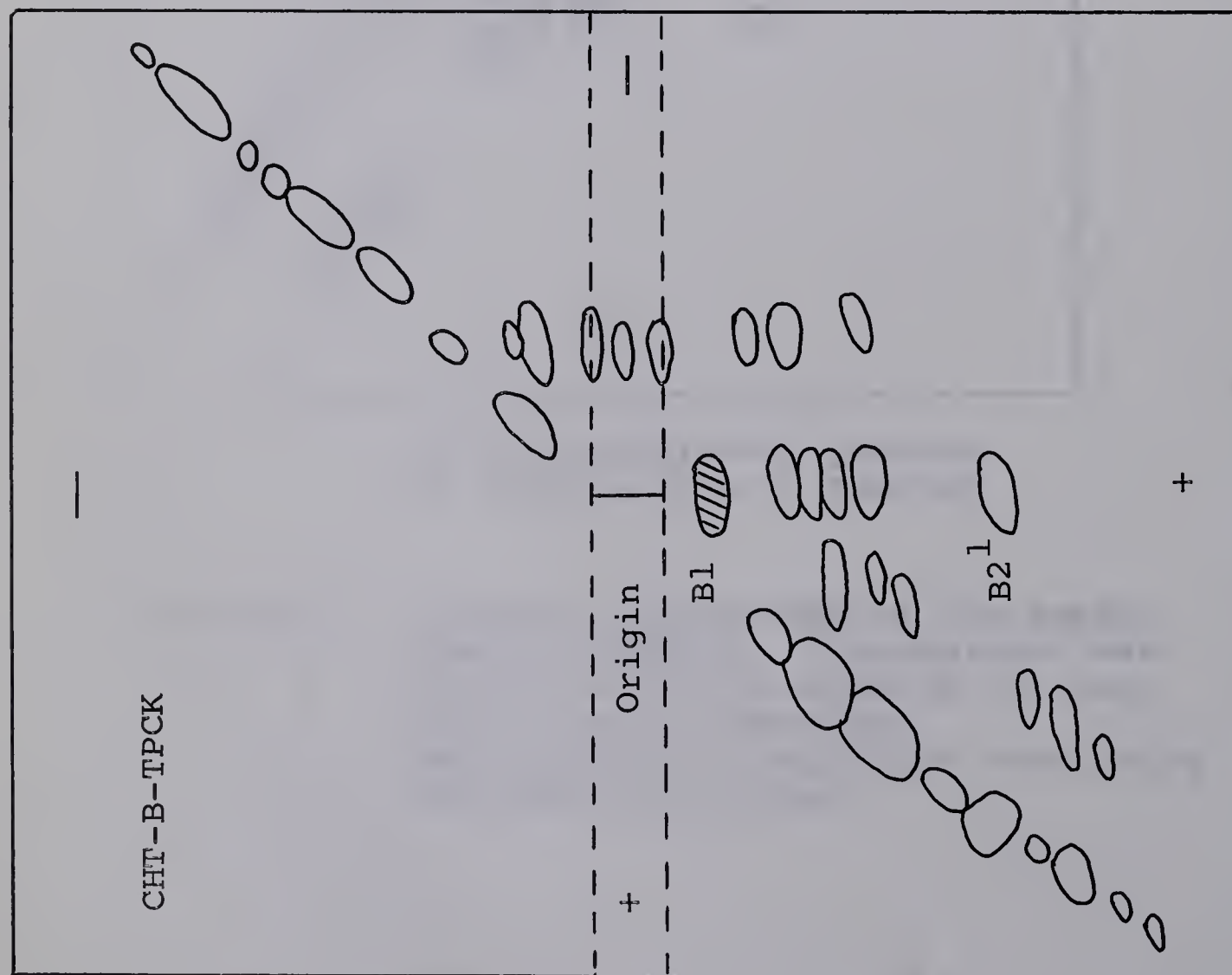
The diagonal peptide maps of the peptic digest of native CHT-A₄ and CHT-A₄ inhibited with L-TPCK and PMCK are shown in Figures 25 and 26 respectively. Histidine peptides detected with the Pauly reagent - diazotized sulfanilic acid, are indicated by cross-hatched areas. Identification of the histidine-40 peptide (A1) and histidine-57 peptide (A2 and A2¹) was based on an amino acid analyses of the purified peptides (section 3) and on the studies of Hartley (11), Brown and Hartley (93, 132) and Smillie and Hartley (16,17,189).

Brown and Hartley (132) have studied in detail the cysteic acid peptides present on the diagonal peptide map of CHT-A₄ (Figure 25). The various peptides migrating off the diagonal have been assigned the sequential number of the cysteic acid residue(s) they contain. Exceptions to this nomenclature are peptides A1 and A2 which contain cysteic-42 and cysteic-58 respectively. The active serine residue-195 and methionine-192 are found in peptide 191-201 in the so called active serine band (peptides 191-201, 220 and 136). Methionine-180 is present

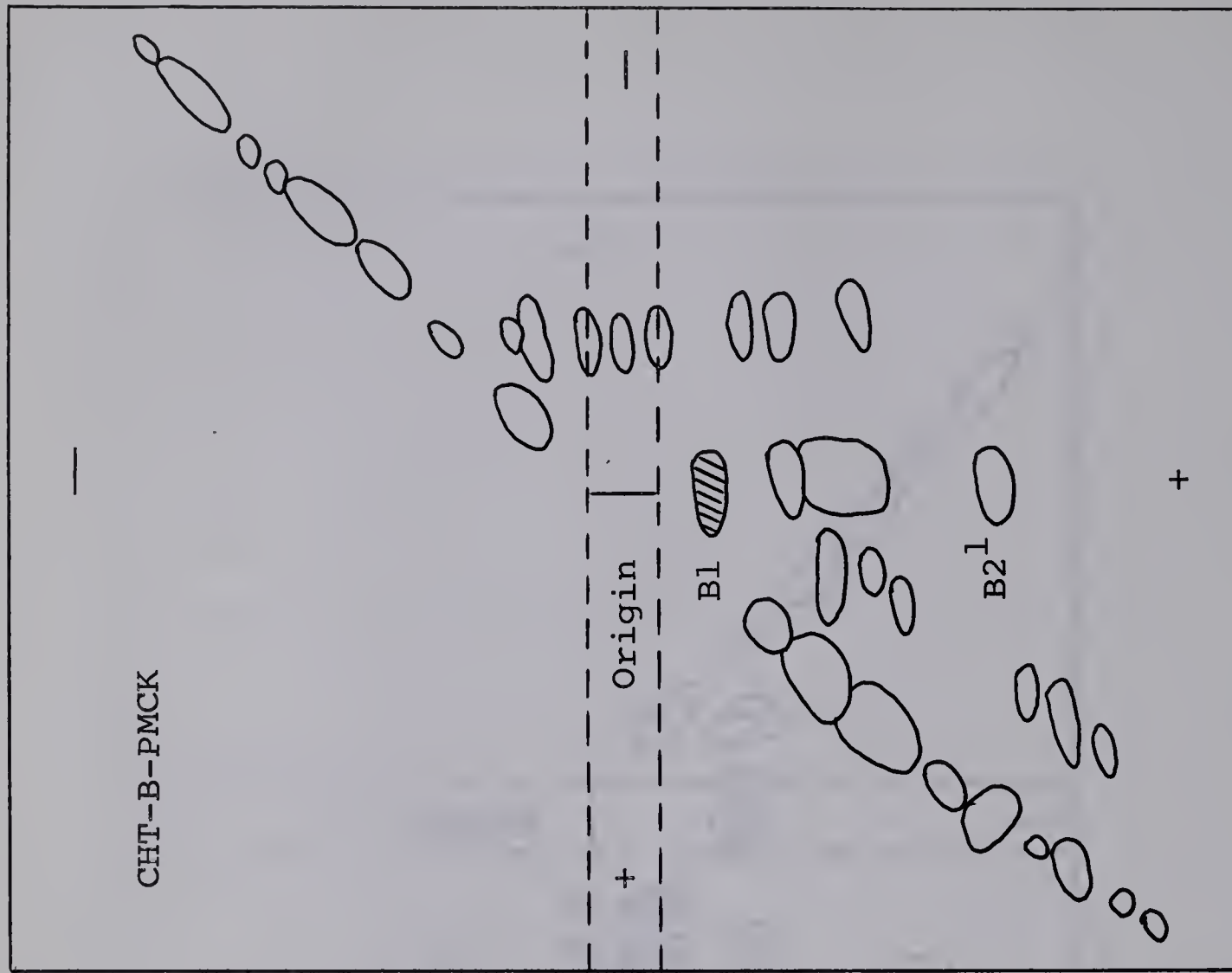


B1 is histidine-40 peptide
B2 is histidine-57 peptide

Figure 27. Diagonal peptide map of the peptic digest of CHT-B. Ionophoresis was at pH 6.5 and 50 volts/cm for one hour in both dimensions. Pauly positive, histidine containing peptides are hatched.



B1 is histidine-40 peptide



B2¹ is alkylated histidine-57 peptide

Figure 28. Diagonal peptide maps of the peptic digests of CHT-B-TPCK and CHT-B-PMCK. Ionophoresis was at pH 6.5 and 50 volts/cm for one hour in both dimensions. Pauly positive, histidine containing peptides are hatched.

in peptides 168 and 168 - 182 well removed from the methionine-192 peptide.

The failure of peptide $A2^1$ (Figure 26) to react with the Pauly reagent suggested N-alkylation of the imidazole ring of histidine-57 (126). The nature of peptide $A2^{11}$ from CHT- A_4 -PMCK was intriguing because it yielded a yellow color with the Pauly reagent. Amino acid analyses of $A2^{11}$ indicated that it was identical to $A2^1$ following acid hydrolysis.

The diagonal peptide maps of CHT-B and CHT-B inhibited with L-TPCK and PMCK are shown in Figure 27 and 28 respectively. The position of the histidine-40 peptide (B1) and the histidine-57 peptide (B2 and $B2^1$) are identical to the positions of peptides A1, A2 and $A2^1$ on the diagonal peptide maps of native and alkylated CHT- A_4 . Peptide $B2^{11}$ corresponding to peptide $A2^{11}$ from CHT- A_4 -PMCK, was not observed on the diagonal of CHT-B-PMCK due to the presence of additional cysteic acid peptides in the same region. The absence of discrete bands in this particular region of CHT-B-PMCK in addition to a characteristic yellow spot obtained with the Pauly reaction, suggested the presence of peptide $B2^{11}$.

It should be noted that the dihistidine cystine peptide of native CHT- A_4 and CHT-B migrates with the neutral band during the first dimension ionophoresis whereas the dihistidine cystine peptide from the alkylated chymotrypsins remained at the origin. The apparent cause of this alteration in mobility could be attributed to two factors:

1. increase in the molecular weight of the peptide due to the TPCK or PMCK substituent, or more likely,

2. a lowering of the pKa of the imidazole group of histidine-57 through inductive effects (-I), resulting in an increase in the net negative charge on the peptide.

Alteration in the mobilities of peptides other than the dihistidine cystine peptides and specifically histidine-57 peptide were not observed on the diagonal peptide maps shown in Figure 26.

(iii) Peptide Purification and Amino Acid Analysis

Purification of the histidine peptides from CHT-A₄ and CHT-B and from the alkylated enzymes was obtained by following a common procedure.

A peptic digest of the native or alkylated chymotrypsins was spotted (1 mg/cm) as a 40 cm band in the center of a Whatman 3MM paper and subjected to ionophoresis at pH6.5, 50 volts/cm for one hour. After location of the dihistidine cystine peptide by staining side strips with the Pauly reagent and cadmium-ninhydrin, the peptide was cut out as a band and sewn on to another sheet of Whatman 3MM paper (origin 10 cm from anode) and further purified by ionophoresis at pH1.8, 75 volts/cm for 30 minutes. Location of the histidine peptide was again obtained by spraying side strips with the Pauly reagent. The Pauly positive band was cut out and oxidized under the conditions as outlined earlier for the diagonal technique. After the band had been dried under vacuum over NaOH for one hour, the oxidized peptides were eluted (234) with water and reapplied a distance of 22 cm from the anode of a sheet of Whatman #1 paper. The peptides were finally purified by ionophoresis at pH3.5, 50

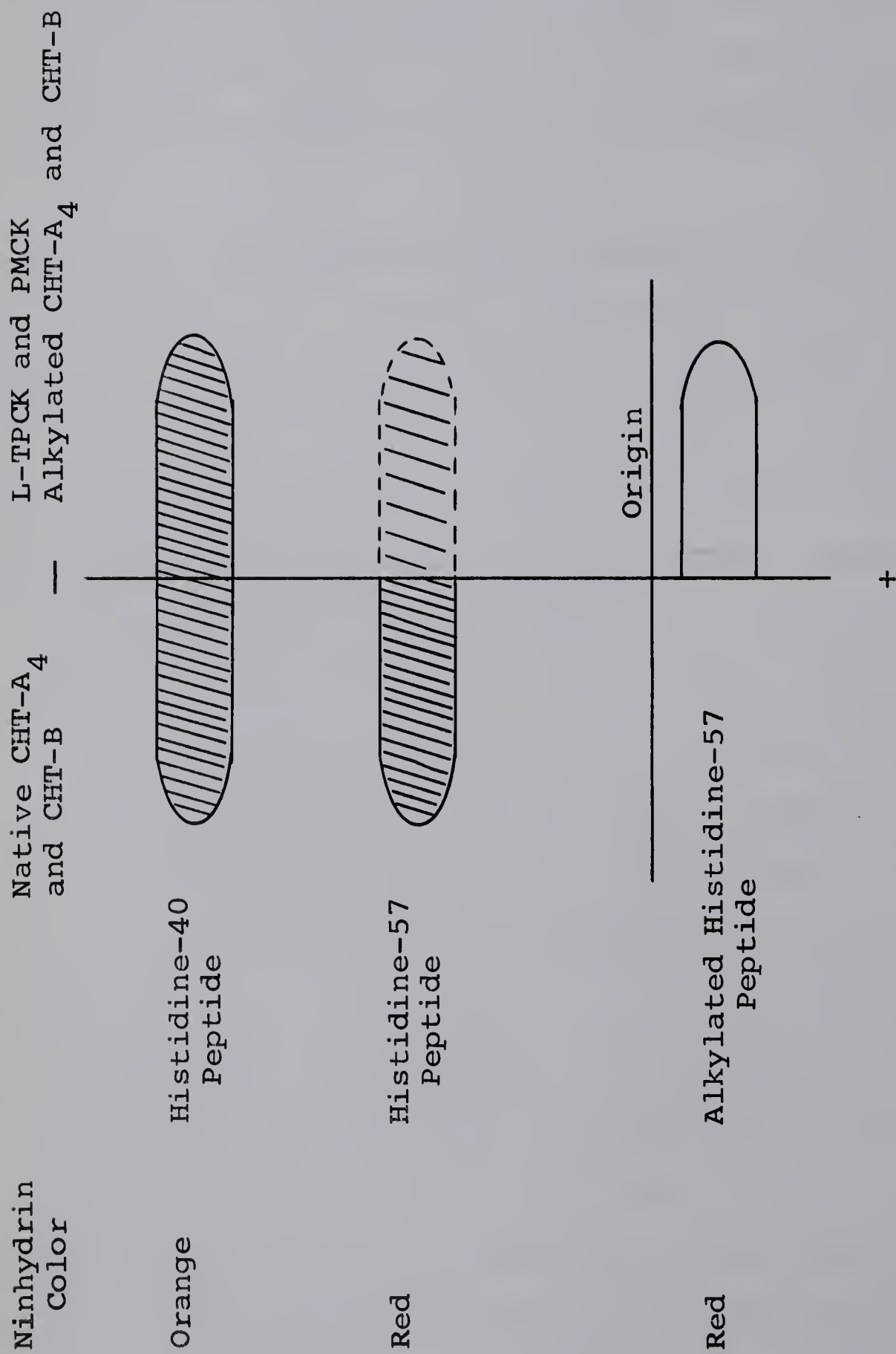


Figure 29. Purification of Histidine-40 and -57 peptides from native and alkylated (L-TPCK and PMCK) chymotrypsins. Ionophoresis was at pH 3.5 and 50 volts/cm for two hours. Pauly positive, histidine containing peptides are hatched.

HISTIDINE-57 PEPTIDE OF CHYMOTRYPSIN-A₄-TPCK

0.3

0.2

0.1

CYSTEIC

ASPARTIC

THREONINE

SERINE

GLYCINE

ALANINE

UNKNOWN

VALINE

200 min

180

160

140

120

100

80

60

40

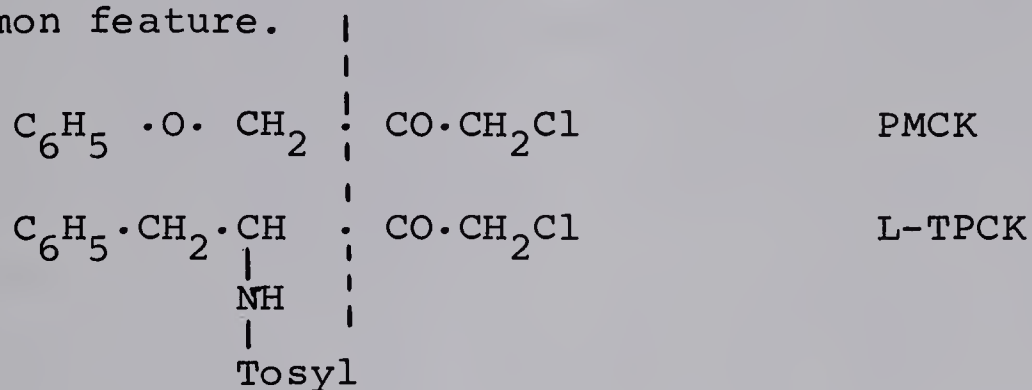
Figure 30. Chromatogram of the amino acid analysis of histidine-57 peptide from CHT-A₄-TPCK.

volts/cm for two hours. Figure 29 shows the purification of histidine-40 and histidine-57 peptides from native and alkylated chymotrypsins.

The histidine-40 and histidine-57 peptides from native and alkylated chymotrypsins were cut out and eluted with water (234) into a 10 x 75 mm pyrex test tube. An equal volume of concentrated HCl was added to the eluents and the peptides were hydrolyzed for 20 hours at 110° in a sealed evacuated tube. Following the removal of the HCl in a dessicator under a vacuum provided by an oil pump, the residue was dissolved in an appropriate volume of 0.2N citrate buffer pH2.2 and subjected to amino acid analysis.

The amino acid chromatogram of the acid hydrolysate of histidine-57 peptide from CHT-A₄-TPCK is shown in Figure 30. A new peak appeared in the vicinity of cystine. Since cystine residues were converted to cysteic acid residues during performic acid oxidation and since histidine was not present in the hydrolysate, the new peak suggested the presence of a modified histidine residue. It should be noted that Smillie and Hartley (105) have observed the presence of a new amino acid on a two dimensional chromatogram of the acid hydrolysate of the performic acid oxidized histidine-57 peptide from CHT-A₄-TPCK. A clue as to the nature of this new derivative was first gleaned from the amino acid chromatogram of the acid-hydrolysate of histidine-57 peptide from CHT-A₄-PMCK. The analyses of the histidine-57 peptide isolated from CHT-A₄ inhibited with two different bifunctional reagents were analogous.

It appeared then that some structural aspect, common to both PMCK and L-TPCK, was leading to the formation of the same unique histidine derivative. Scrutiny of the structure of the reagents revealed that the chloromethyl ketone moiety was a common feature.



Examination of Figures 25 and 26 indicates that peptide A2¹ is very acidic and possesses a mobility some 2.5-fold greater than the native A2 peptide. Two possibilities arise which could account for the acid nature of the alkylated histidine peptide, A2¹:

1. loss of a positive charge on the peptide, or
2. acquisition of a negative charge.

If it is assumed that the pKa of the imidazole group could be reduced to ~ 5.5 by an uncharged electron-withdrawing substituent, then the alkylated histidine-57 residue would be predominantly unionized at pH6.5 and thereby devoid of a positive charge. An estimate of the net charge would have placed a value of -2 on the peptide, which appeared to be insufficient to contribute the observed mobility. Moreover, the net charge on such an alkylated histidine-57 peptide would be essentially the same as the native histidine-57 peptide at pH3.5. The observed mobilities of the peptides at pH3.5 (Figure 29) disprove the possibility that an uncharged electron-withdrawing

TABLE IX

Comparison of the net charge and distance migrated of native and alkylated histidine peptides on pH 3.5 and pH 6.5 ionophoresis

Peptide	pH 3.5		pH 6.5	
	Net Charge	Distance Migrated ^a (cm)	Net Charge	Distance Migrated ^a (cm)
Histidine-40 (7 Residues)	A1	0.3 ^b	16.0 (-)	-0.9 ^c 6.0 (+)
Histidine-57 (10 Residues)	A2	0.5 ^d	10.5 (-)	-1.2 ^e 7.2 (+)
3-Carboxymethyl Histidine-57	A2 ¹	-0.1 ^f	2.5 (+)	-2.7 ^g 10.5 (+)

- a. Measured from neutral region. (+) or (-) refers to migration towards anode or cathode, respectively. (pH 3.5, 50 V/cm 2 hours; pH 6.5, 50 V/cm, 1 hour)
- b. Based on pKa = 3 for the C-terminal leucine residue
- c. Based on A pKa = 5.6 for the imidazole group of an N-terminal histidine (237)
- d. Based on pKa values of 3.4 and 4.7 for the β -COOH and α -COOH of C-terminal aspartic acid. (237)
- e. pKa \sim 7.2 of imidazole of histidine due to the field effect of the adjacent cysteic acid residue.
- f. pKa of the 3-carboxymethyl group estimated to be \sim 3.7.
- g. pKa of histidine group estimated to be 6.2.

substituent was present on the imidazole group of histidine-57.

The second alternative of introducing a negatively charged substituent on to the imidazole ring appeared to be quite attractive. Peracids (RCO_3H) are known to react with ketones to form esters following rearrangements. (Hassall (238)). Subsequent hydrolysis of the ester could readily yield an acid. Since ketone moieties existed in the TPCK - and PMCK - histidine derivatives, it appeared highly probable that this group could be rearranged to yield an ester. Hydrolysis of the resulting ester during the two hour oxidation stage was highly probable since the paper strips were thoroughly impregnated with formic and performic acid vapours. These deductions pointed towards the formation of a carboxymethyl derivative of histidine.

Crestfield et al. (230) have shown the elution position of 1-carboxymethylhistidine to be between glutamic acid and proline and 3-carboxymethylhistidine to be at the position of cystine on the amino acid analyses system of Spackman, Moore and Stein (239). The position of the unknown peak on the amino acid chromatogram (Figure 30) of TPCK-histidine-57 peptide corresponded precisely with the position of 3-carboxymethylhistidine (3-CM histidine).

Comparison of the net charge and the migration distances of the native histidine-40 and -57 and the alkylated (3-carboxymethyl histidine-57 peptide at pH 3.5 and pH 6.5 provided additional evidence for the existence of a 3-carboxymethyl derivative (Table IX). Estimation of the net charge was based

TABLE X

Amino Acid Analyses of Histidine Peptides from
Native and Alkylated CHT-A₄.

<u>Histidine-40 Peptide</u>			
<u>Amino Acid</u>	<u>CHT-A₄^a</u>	<u>CHT-A₄-TPCK</u>	<u>CHT-A₄-PMCK</u>
Histidine	1	0.88	0.80
Cysteic Acid	1	0.94	0.95
Serine	1	0.95	1.05
Glycine	2	1.93	1.90
Leucine	1	1.00 ^b	1.00 ^b
Phenylalanine	1	0.92	0.90
<u>Histidine-57 Peptide</u>			
<u>Amino Acid</u>	<u>CHT-A₄^a</u>	<u>CHT-A₄-TPCK</u>	<u>CHT-A₄-PMCK</u>
Histidine	1	-	-
Cysteic Acid	1	0.96	.93
Aspartic Acid	1	1.00	1.03
Threonine	2	1.92	1.83
Serine	1	0.96	0.93
Glycine	1	1.00	1.07
Alanine	2	2.00 ^c	2.00 ^c
3-Carboxymethyl Histidine	-	0.96 ^d	0.90 ^d
Valine	1	0.96	1.00

a. Data of Brown and Hartley (93, 132)

b. Arbitrarily taken as 1.0 residue

c. Arbitrarily taken as 2.0 residues

d. Calculated from an integration constant of 43.6 for glycine. (Crestfield et al. (230))

TABLE XI

Amino Acid Analyses of Histidine Peptides from
Native and Alkylated CHT-B.

<u>Histidine-40 Peptide</u>			
<u>Amino Acid</u>	<u>CHT-B^a</u>	<u>CHT-B-TPCK</u>	<u>CHT-B-PMCK</u>
Histidine	1	0.88	0.90
Cysteic Acid	1	1.01	1.10
Serine	1	0.87	0.75
Glycine	2	1.98	2.10
Leucine	1	1.00 ^b	1.00 ^b
Phenylalanine	1	0.96	0.93
<u>Histidine-57 Peptide</u>			
<u>Amino Acid</u>	<u>CHT-B^a</u>	<u>CHT-B-TPCK</u>	<u>CHT-B-PMCK</u>
Histidine	1	-	-
Cysteic Acid	1	0.98	0.91
Aspartic Acid	1	1.05	0.98
Threonine	2	1.79	1.62
Serine	1	0.91	0.72
Glycine	1	1.12	1.13
Alanine	2	2.00 ^c	2.00 ^c
3-Carboxymethyl Histidine	-	1.02 ^d	0.95 ^d
Valine	1	0.98	0.98

a. Data of Smillie and Hartley (17, 189)

b. Arbitrarily taken as 1.0 residue

c. Arbitrarily taken as 2.0 residues

d. Calculated from an integration constant of 43.6 for glycine (Crestfield et al. (230))

HISTIDINE - 57 PEPTIDE OF CHYMOTRYPSIN - A₄ - PMCK

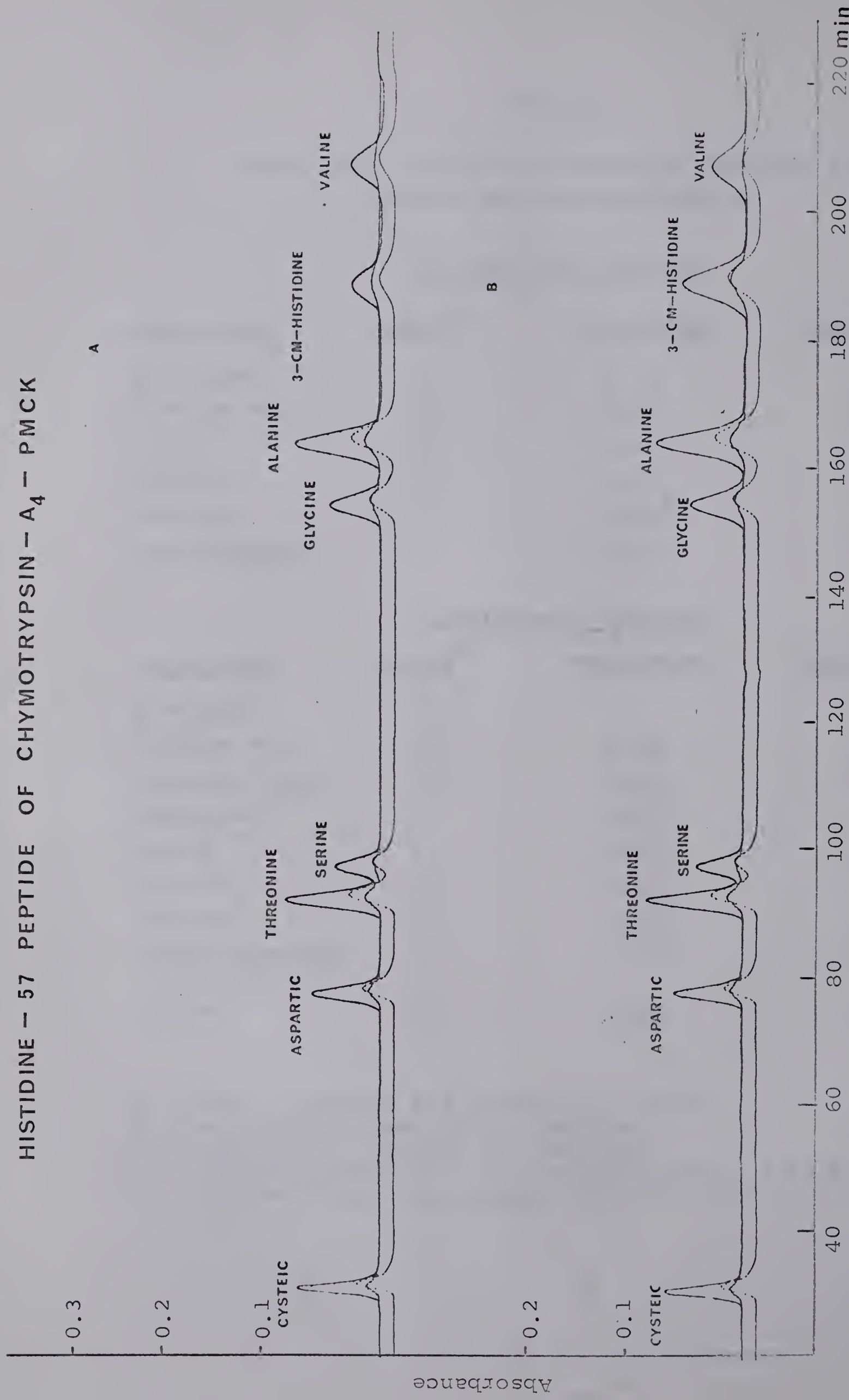


Figure 31 Chromatograms of the amino acid analysis of histidine-57 peptide from CHT-A₄-PMCK in the absence (A) and in the presence (B) of authentic 3-carboxymethylhistidine (3-CM-histidine).

largely on the work of Greenstein (237) on dihistidine and diaspartic acid peptides.

Amino acid analyses of histidine-40 and -57 peptides from native and alkylated CHT-A₄ and CHT-B are presented in Tables X and XI, respectively. The insertion of 3-CM histidine in the tables was based on studies to be presented in the following section. The histidine-40 peptides isolated from native and alkylated chymotrypsins were found to be identical. However, histidine-57 peptides from L-TPCK and PMCK alkylated CHT-A₄ and CHT-B were not analogous to the histidine-57 peptides from the native chymotrypsins. In all cases, the histidine residue in the alkylated peptides was recovered as its 3-carboxymethyl derivative.

(iv) Characterization of the 3-carboxymethyl Histidine-57

Although the new peak present on the amino acid chromatogram of histidine-57 peptide from L-TPCK and PMCK alkylated chymotrypsin was strongly suggestive of 3-CM histidine, unequivocal evidence was required. To obtain such evidence, 3-CM histidine was synthesized from acetyl-L-histidine and iodoacetic acid according to the method of Crestfield, Moore and Stein (230). 3-CM histidine, isolated following one crystallization from 80% ethanol (Cal: C,43.05; H,4.98; N, 23.30. Found: C, 41.27; H, 5.80; N,17.85) was dissolved in 0.2N citrate buffer pH2.2 and suitable aliquots were analyzed. Figure 31 depicts the amino acid chromatogram obtained when duplicate samples of the acid hydrolysate of histidine-57 peptide from CHT-A₄-PMCK were analyzed in the absence (A) and

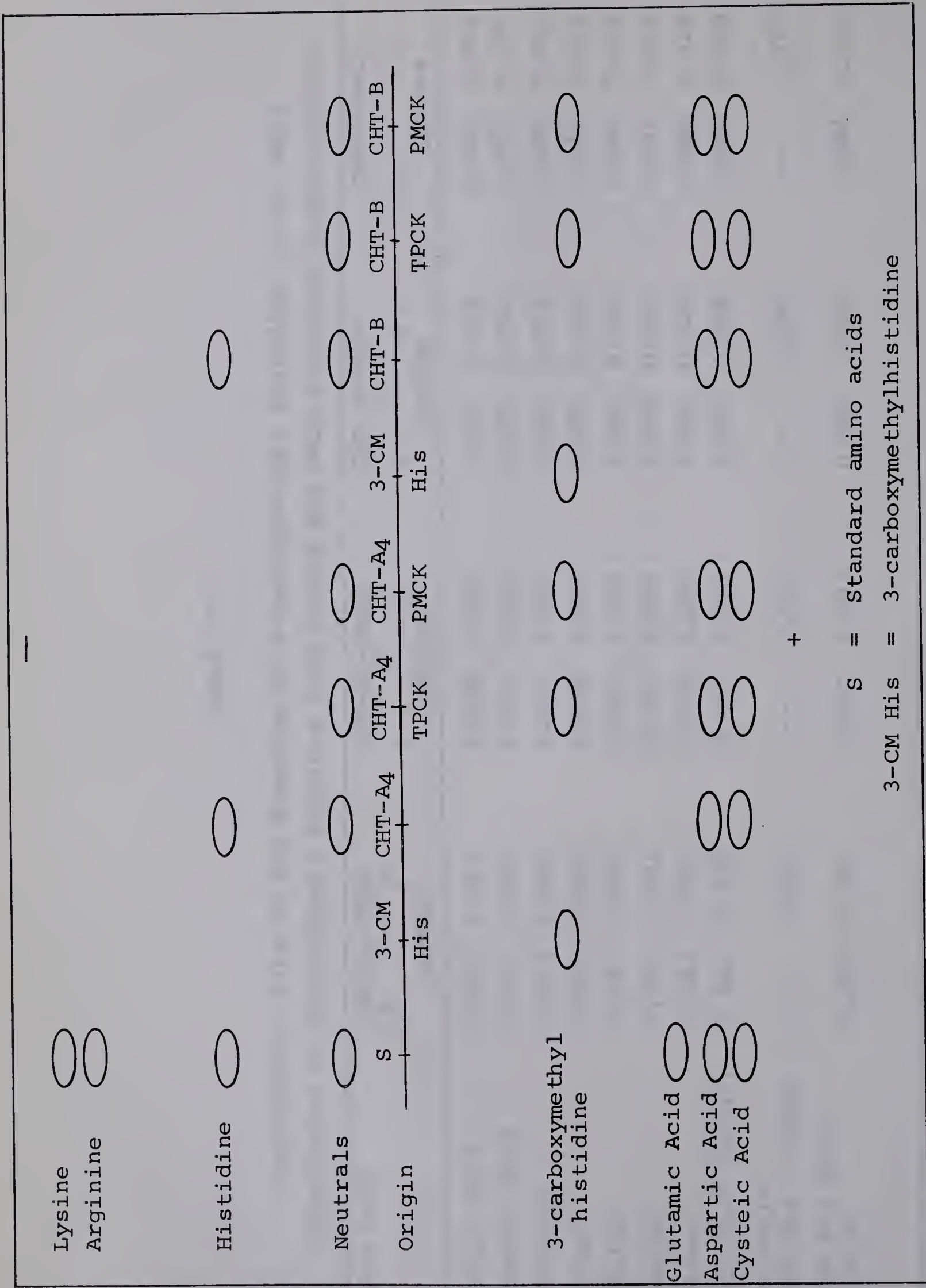
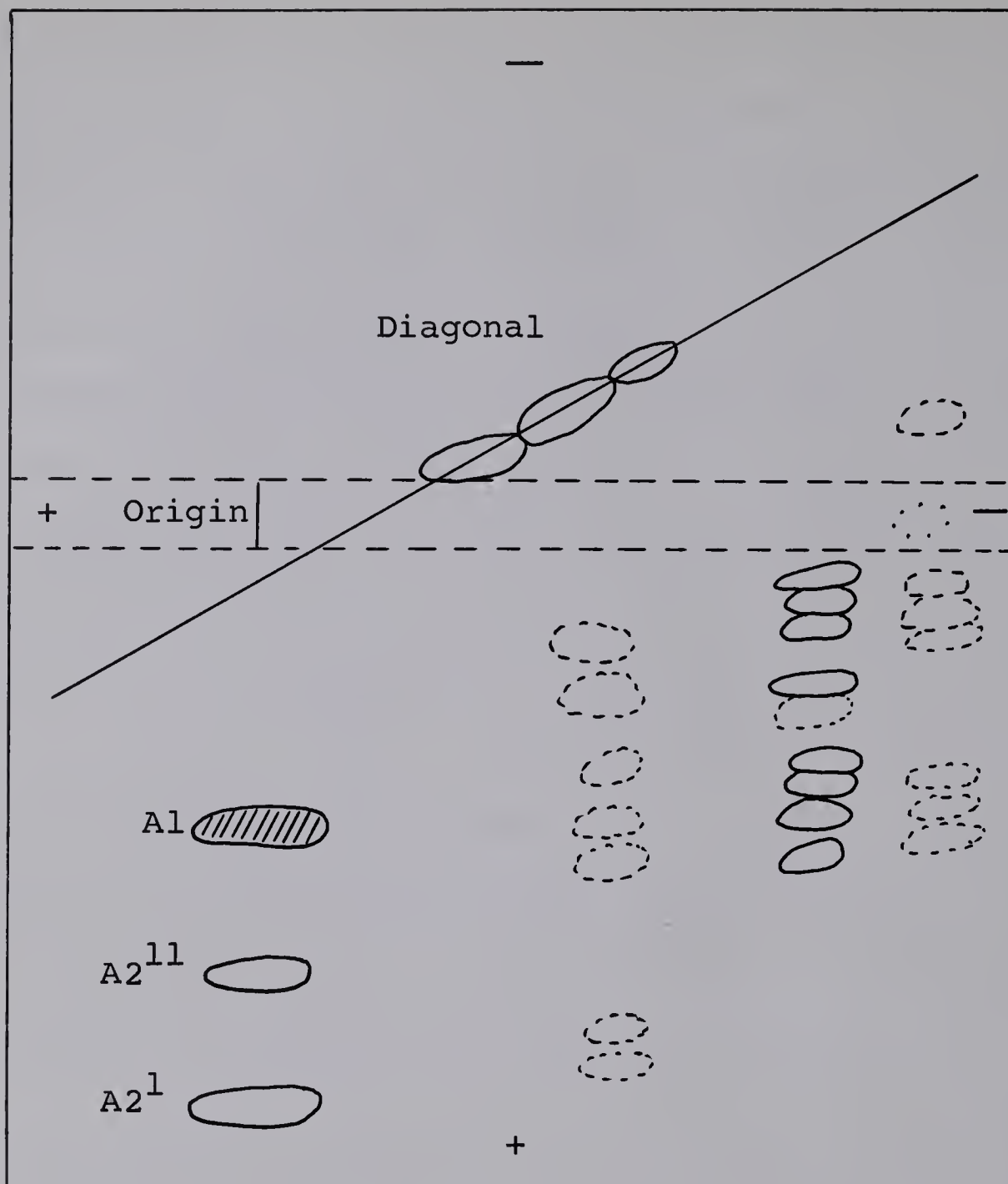


Figure 32. Amino acid analysis of Histidine-57 peptides from native and alkylated Chymotrypsins. Ionophoresis was at pH 6.5 and 50 volts/cm for 50 minutes.

in the presence (B) of a known quantity of authentic 3-CM histidine. The authentic 3-CM histidine is seen to be eluted in precisely the same position as the new peak in the histidine-57 analysis. Similar experiments conducted with the amino acid hydrolysates of histidine-57 peptides from CHT-A₄-TPCK, CHT-B-TPCK, and CHT-B-PMCK were identical to the chromatograms presented in Figure 31. Quantitative data shown in Table XII indicate that the recovery of 3-CM histidine contributed by the peptide, in the absence (Column 1) and in the presence (Column 2) of added synthetic 3-CM histidine is in excellent agreement with the duplicate runs.

Crestfield et al. (230) have shown that 1-CM histidine was eluted between glutamic acid and proline on the chromatographic system of Spackman et al. (239). No evidence of 1-CM histidine was obtained from the analyses of histidine-57 peptides.

Additional support for the occurrence of 3-CM histidine in the alkylated histidine-57 peptides was amply furnished by qualitative amino acid analyses utilizing ionophoresis at pH6.5. This system was particularly appropriate since histidine (estimated net charge 0.7) and 3-CM histidine (estimated net charge -0.3) migrate midway between the origin and the basics and the origin and acidics respectively. Loss of a histidine residue with concomitant formation of a 3-CM histidine residue was easily detected by ionophoresis at pH6.5 under these conditions. The amino acid ionogram presented in Figure 32 again confirms the presence of 3-CM histidine in the histidine-57



A1 is histidine-40 peptide
A2¹ is 3-carboxymethylhistidine-57 peptide.
A2¹¹ is the phenoxymethyl ester of 3-carboxymethylhistidine-57 peptide.

Figure 33. Diagonal peptide map of the peptic digest of CHT-A₄-PMCK. Ionophoresis was run at pH 6.5 and 50 volts/cm. First dimension: 3 hours. Second dimension: 2 hours. Pauly positive, histidine containing peptides are hatched.

peptides of L-TPCK and PMCK alkylated chymotrypsins.

Attempts to prepare L-TPCK - histidine and PMCK - histidine derivatives from acetyl-histidine, trifluoroacetyl-histidine and free histidine were unsuccessful. Heinrickson et al. (231) has observed that the extent of reaction between histidine and bromoacetate at pH5.5 was always less than 5%. The second order rate constant of the reaction with L-histidine on N-1 or N-3 was found to be $8.6 \times 10^{-6} \text{ l moles}^{-1} \text{ sec}^{-1}$. Furthermore, a notable decrease in the rate of reaction with L-histidine was observed when the chain length of the α -bromo acid was extended from two to three or more carbon atoms. Korman and Clerke (240) found that in the haloacetate series the order of reactivity with histidine was $\text{Br} > \text{I} \gg \text{Cl}$. The inability to form N1 or N3 alkylated histidine derivatives with L-TPCK and PMCK appears to be due to the inherent unreactive nature of the chloromethyl ketones (relative to bromomethyl ketones) coupled with the limited solubility of these reagents in aqueous ethanol solutions.

(c) Discussion

Inhibition of CHT-A₄ and CHT-B with the bifunctional reagents L-TPCK and PMCK has been shown to be predominantly associated with the alkylation of the nitrogen 3 position of the imidazole ring of histidine-57. Through determination of the methionine sulfone content, both reagents have been implicated in the partial S-alkylation of a methionine residue. A diagonal peptide map of CHT-A₄-PMCK on Whatman #1 paper, (Figure 33) obtained by running the initial pH6.5 ionophoresis

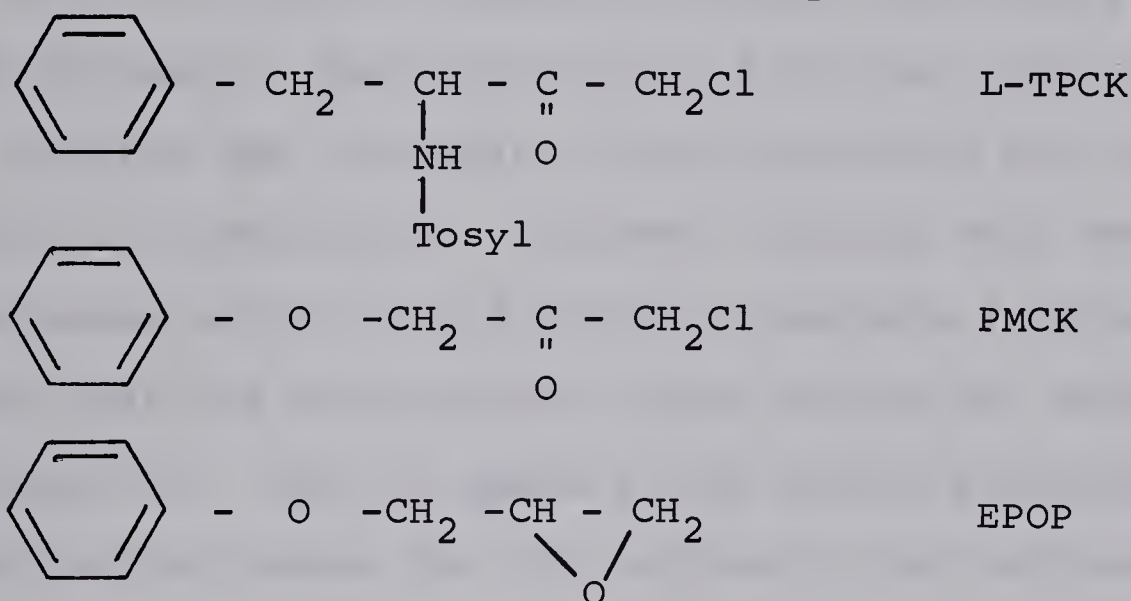
for three hours and the second dimension ionophoresis for two hours, revealed traces of peptides migrating towards the cathode in advance of the active serine band. Studies to be presented in Section 3 indicated that the traces of this basic band were due to the presence of a positive charge contributed by the sulfonium salt of methionine-192. It can be concluded that the loss of methionine sulfone residues and the presence of trace amounts of basic active center peptides of CHT-A₄-PMCK were the result of the S-alkylation of methionine-192 by PMCK. Although L-TPCK appears to alkylate CHT-A₄ in an analogous manner to PMCK, diagonal peptide maps obtained for CHT-A₄-TPCK under similar conditions to those just outlined for CHT-A₄-PMCK, failed to reveal the presence of a basic active center band. Possibly the instability of the sulfonium salt formed by the reaction between L-TPCK and methionine accounts for the apparent absence.

Alkylation of submolar amounts of methionine (possibly residue 192) has been demonstrated with D-TPCK and N-methyl-L-TPCK. These reagents were not capable of alkylating histidine -57.

Of particular interest was the finding that L-TPCK and PMCK formed methionine sulfonium salt when incubated with CHTG-A. Studies of Vaslow and Doherty (18) and Neurath and Deranleau (19, 241) have indicated that the substrate binding site is functional in CHTG-A and is similar to the binding site in the active enzyme. The present observations support the existence of a binding site in the zymogen since the bifunctional reagents could be readily bound to CHTG-A and

subsequently lead to the S-alkylation of a methionine residue nearby.

Brown and Hartley (42) have found that 1,2-epoxy-3-phenoxypropane (EPOP), a reagent similar to PMCK, stoichiometrically inhibits CHT-A₄ by virtue of the S-alkylation of methionine-192. From a comparison of these compounds (see below) it is possible to draw certain conclusions concerning the relative importance of their structural features in determining the site of alkylation.



Firstly, the asymmetric carbon and the tosylamido group are unnecessary for alkylation of histidine-57. However, if these are present, then the configuration must be of the L-form for proper steric fit. Methylation of the tosylamido group of L-TPCK sterically hinders the alkylation of histidine-57. Secondly, the replacement of the phenylmethyl group by the phenoxy radical is not effective in directing the alkylation to the methionine. However, the proper spacial relationship between the aromatic and the chloromethyl ketone which leads to histidine alkylation is maintained by the phenoxy radical

of PMCK. It is clear that the major factor determining the site of attack is the nature of the alkylating group. Apparently, the steric requirements for the approach of an alkylating group to the nitrogen 3 position of histidine-57 are sufficiently restrictive to make reaction between an epoxide and the imidazole nitrogen impermissible. The chloromethyl ketone derivatives meet these requirements, in whole or in part, and alkylation occurs. Alternatively, the differences in sites of alkylation could conceivably be due to a difference in the reactivities of the two alkylating groups towards the thioether of methionine-192 and the imidazole of histidine-57. However, studies with PMCK and an homologous series of bifunctional reagents (section 3) indicate that the chloromethyl ketone moiety can alkylate the methionine-192. Thus it appears that steric requirements are the controlling factor for the approach of an alkylating group to the nitrogen 3 of histidine-57.

The stoichiometry of the alkylation of CHT-A₄ with L-TPCK has been shown by Schoellman and Shaw (104), using labeled ¹⁴C-TPCK, to be 0.96 moles of L-TPCK/mole of CHT-A₄. This figure correlated well with the observed loss of histidine residues. However, studies presented herein indicate that a methionine is also alkylated by L-TPCK and thus would necessitate the presence of ~1.4 moles of L-TPCK/moles of CHT-A₄.

The discrepancy could arise through decomposition of the sulfonium salt, with loss of the tosylphenylalanine moiety, prior to determination of the stoichiometry of the reaction.

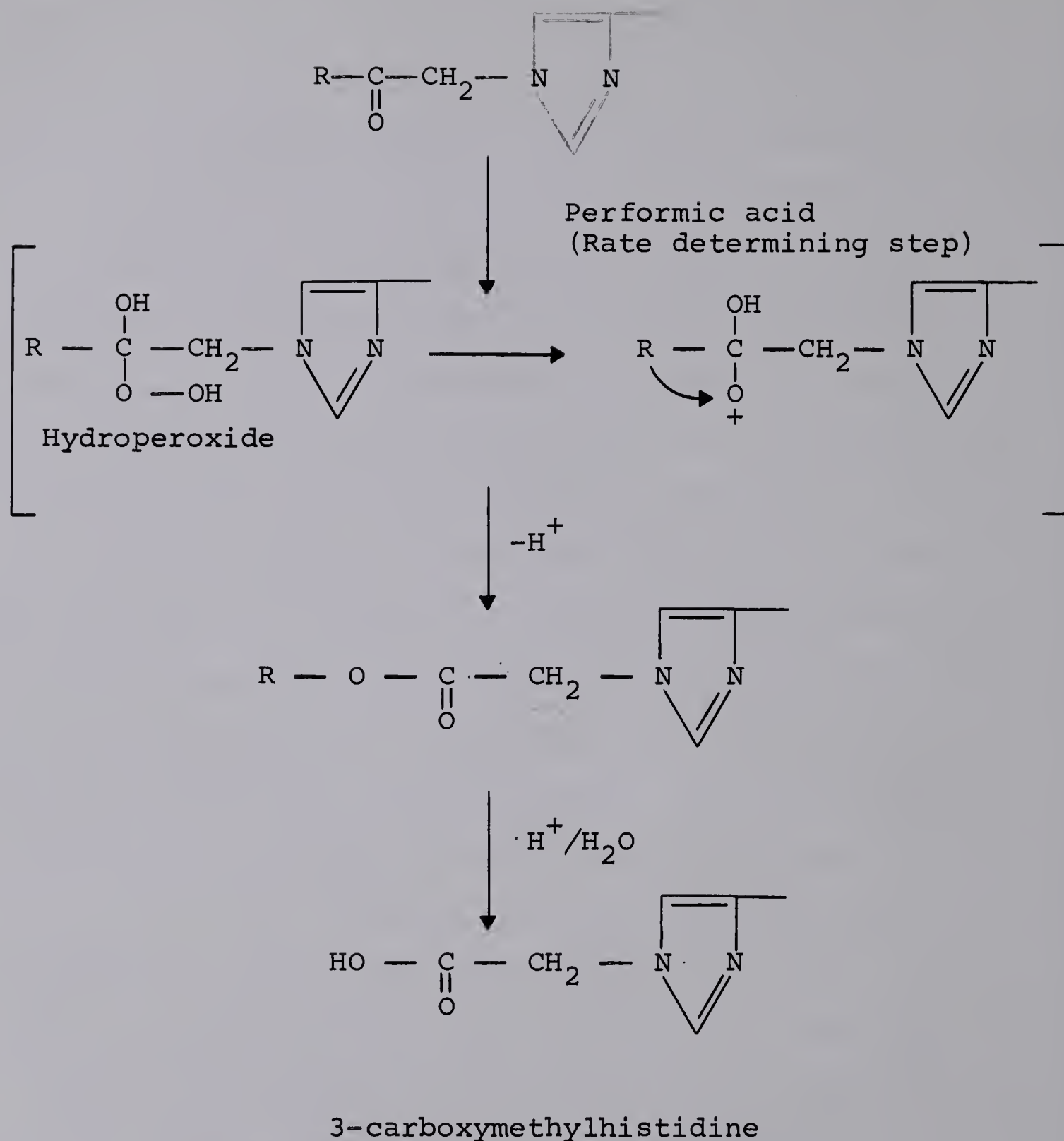
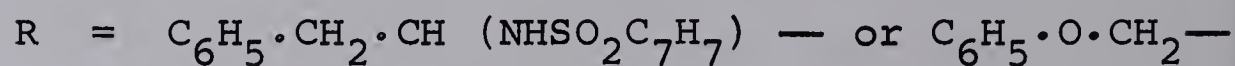


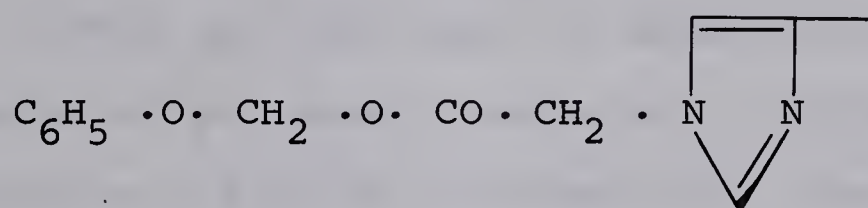
Figure 34. Performic acid rearrangement of TPCK- and PMCK- histidine-57 to yield 3-carboxymethyl histidine-57.



Support for the instability of the TPCK-methionine sulfonium salt may be procured from the apparent absence of a basic active serine band on the diagonal peptide map obtained by subjecting the peptic digest to prolonged ionophoresis in both dimensions.

The 3-CM histidine isolated from acid hydrolysis of the histidine-57 peptide from L-TPCK and PMCK alkylated chymotrypsin was formed through a classical Baeyer-Villiger peracid rearrangement (242) of a ketone to an ester. The site of the rearrangement was the ketone moiety of TPCK-histidine and PMCK-histidine derivatives. The reaction of the ketones with performic acid (HCOOOH) likely proceeds via a mechanism suggested by Criegee (243) and extended by Doering and Dorfmann (244) (Figure 34). The rate - determining step is the acid catalyzed addition of the peracid to the carboxyl group to yield a hydroperoxide. This complex dissociates (probably in a concerted manner) to give an electron-deficient oxygen atom which rapidly rearranges with cleavage of a C-C bond and migration of an alkyl group to form an ester. The migratory aptitude of an alkyl group is normally proportional to its capacity for electron release (i.e. ability to delocalize a positive charge) and is indicated by the following series: tert-alkyl > cyclohexyl ~ sec-alkyl ~ benzyl ~ phenyl > primary alkyl > methyl. Clearly, the groups migrating in the present study were the L-1-tosylamido-2-phenylethyl moiety of L-TPCK and the phenoxymethyl moiety of PMCK. The ester formed as a result of the peracid rearrangement in TPCK-histidine-57 peptide was completely hydrolysed during the performic oxidation

as witnessed by the presence of a single modified 3-CM histidine-57, peptide ($A2^1$) on the diagonal peptide maps (Figures 26 and 28). However, the ester resulting from the peracid rearrangement of PMCK-histidine-57 peptide was incompletely hydrolysed during oxidation and appeared as two peptides $A2^1$ (3-CM histidine-57) and $A2^{11}$ on the diagonal peptide maps. Curiously, peptide $A2^{11}$ yielded a yellow-colored spot with the Pauly reagent. Purification of peptide $A2^{11}$ was obtained by a combination of ionophoresis runs at pH 6.5 and pH 1.8. Amino acid analyses of the band was synonymous with peptide $A2^1$. Thus, it can be concluded that peptide $A2^{11}$ represents the ester intermediate shown below.



The mobility of $A2^{11}$, relative to the native histidine-57 peptide, is in accordance with an increase in the net negative charge of the peptide resulting from the lowering of the imidazole pKa through inductive effects (-I).

It is interesting to note that performic acid oxidation employed in the vapour state (desiccator) was sufficiently mild not to rupture the imidazole nucleus. However, the rigorous oxidation conditions prevalent in the method outlined by Moore (235) destroyed histidine.

The reagents L-TPCK and PMCK are truly bifunctional in nature and require an active chymotrypsin before alkylation of histidine-57 will occur. Schoellman and Shaw (104) have demonstrated that L-TPCK will not react with DIP-CHT- A_4 or

CHT-A₄ which had been denatured in 8M urea. Similarly, studies in this laboratory have shown that urea denatured chymotrypsin is not susceptible to PMCK alkylation. Histidine residues in CHTG-A are not altered by L-TPCK or PMCK but the formation of methionine sulfonium salts arising from S-alkylation of the thioether group have been demonstrated. The inhibition of CHT-A₄ with L-TPCK and PMCK is delayed in the presence of a competitive inhibitor-β-phenylpropionate — thus indicating that both reagents are bound to the active site of the enzyme (104, 109). The reagents are highly selective and will not inhibit trypsin — an enzyme closely related to the chymotrypsins but differing in its specificity (12,150,151).

L-TPCK and PMCK must be considered as chymotryptic reagents and not merely reagents to alkylate histidine residues in proteins. Failure to accept this view has led to misleading conclusions concerning the role of histidine in thrombin (251).

Enzymes possessing specificities similar in nature to the chymotrypsins would be anticipated to be susceptible to these reagents. Porcine chymotrypsin C recently studied by Folk et al. (252, 253) is an obvious example. Gibson and Dixon (245, 246) have observed that two "serine" proteases, isolated from the glandular tissue of the sea anemone, possessed strong activity in hydrolyzing ATEE and were rapidly inactivated by L-TPCK. Streptococcal proteinase, a sulfhydryl enzyme, readily hydrolyses the synthetic substrate carbobenzoxy-PHE-PHE (246). This, in addition to the dependency of the hydrolysis on a group with $pK_a \sim 6.4$, suggests that the enzyme may be inhibited

by L-TPCK.

Following the design which lead to the success of L-TPCK with CHT-A₄, Shaw et al. (143, 248) have synthesized chloromethyl ketone derivative from N-TOSYL-L-lysine (TLCK) to serve as a bifunctional reagent for trypsin. TLCK was demonstrated to specifically alkylate the nitrogen 3 position of a histidine residue by employing the performic acid rearrangements discussed in this thesis. The particular histidine alkylated was found to be residue 57 (254) and corresponds to the histidine-57 residue alkylated by L-TPCK and PMCK in the chymotrypsins (105, 109).

3. Elucidation of the Mode of Inhibition of CHT-A₄ with a Homologous Series of Bifunctional Reagents.

(a) Methods

(i) Preparation of CHT-A₄ Inhibited with a Homologous Series of Bifunctional Reagents.

CHT-A₄ (100 mg) was dissolved in 250 ml of 0.05 M tris-HCl buffer, 0.05 M CaCl₂, at pH 7.5 containing 6 ml of ethanol. Two ml of the solution were removed as a blank α -chloroacetophenone (CA, C₆H₅·CO·CH₂Cl, 154.6 mg, molar ratio 250/1), anisoyl chloromethyl ketone (ACK, CH₃O·C₆H₄·CO·CH₂Cl, 24.4 mg, molar ratio 33/1), benzyl chloromethyl ketone (BCK, C₆H₅·CH₂·CO·CH₂Cl, 136 mg, molar ratio 200/1), or β -phenylethyl chloromethyl ketone (β PECK, C₆H₅·CH₂CH₂·CO·CH₂Cl, 146 mg, molar ratio 200/1) in 6.5 ml of ethanol was slowly added to the CHT-A₄ solution. Final ethanol concentration was 5%. The solution was stirred with the aid of a magmix for 41 hours (except in the case of β PECK where a 21 hour incubation was employed) at room temperature. In

preparation for assays of enzyme activity, a 100 μ l aliquot was diluted in 700 μ l of 5×10^{-3} M HCl at 0°. Appropriate aliquots of the HCl solution, assayed against ATEE, yielded the following residual activities: CHT-A₄-CA, 15%; CHT-A₄-ACK, 20%; CHT-A₄-BCK, 35%; and CHT-A₄- β PECK, 3%. The enzyme solution was adjusted to pH 3 with 1 M HCl and dialysed for 24 hours against 3 x 6 litres of 10^{-3} M HCl at 3°. Approximately 90 mg of inhibited CHT-A₄ were recovered after the samples were freeze dried. The material was stored at -20° until required.

(ii) Amino Acid Analyses and Methionine Sulfone Analyses.

Preliminary amino acid analyses of the alkylated chymotrypsins, performed as outlined in section 2, revealed that sub-molar quantities of histidine and methionine were alkylated. In order to obtain a more reliable estimate of the extent of histidine and methionine modification, amino acid analyses were performed in triplicate on three-times the usual amount of protein.

Alkylated CHT-A₄ (10 mg) was dissolved in 3.34 ml of deionized water. Three-1 ml aliquots (each containing 3 mg of protein) were removed and added to three-1 ml aliquots of concentrated hydrochloric acid. Subsequent preparations for hydrolysis and treatment of the hydrolysate prior to analysis were carried out as described earlier. Amino acid analyses were performed on 1 ml aliquots of the hydrolysate (final volume 2.5 ml). In order to obtain adequate separation of

TABLE XIII

Amino Acid Analyses of CHT-A₄ Inhibited with a
Homologous Series of Bifunctional Reagents

Amino Acid	CHT-A ₄ ^a CA	CHT-A ₄ ^a ACK	CHT-A ₄ ^a BCK	CHT-A ₄ ^a βPECK	CHT-A ₄ ^b
Lysine	14.0	14.0	13.6	13.8	14
Histidine	2.0	2.0	1.8	1.6	2
Arginine	2.9	2.9	3.0	2.9	3
Aspartic Acid	21.6	21.3	21.0	20.9	22
Threonine	-	20.3 ^e	20.2 ^e	20.3 ^e	22
Serine	-	22.6 ^e	21.0 ^e	21.0 ^e	27
Glutamic	14.9	14.6	14.6	15.0	15
Proline	9.0	10.2	10.2	9.7	9
Glycine	22.8	22.2	22.4	22.4	23
Alanine	22.0 ^c	22.0 ^c	22.0 ^c	22.0 ^c	22
Half-cystine	8.7	5.5	6.6	7.8	10
Valine	22.4	21.9	22.1	22.5	23
Methionine	1.6 ^d	1.6 ^d	1.9 ^d	2.0 ^d	2
Isoleucine	9.7	9.8	9.6	9.6	10
Leucine	18.8	18.7	18.3	18.4	19
Tyrosine	3.7	3.5	3.6	3.6	4
Phenylalanine	5.6	5.6	5.6	5.7	6
Homoserine Lactone	+	+	-	-	

a. Average of 3 amino acid analyses

b. Data of Hartley (11) corrected for amino acids lost upon activation.

c. Arbitrarily taken as 22.0 residues

d. Sum of methionine and methionine sulfoxides

e. Not corrected for hydrolytic destruction.

lysine and histidine, the 20 cm, rather than the 11 cm basic column was utilized.

Methionine sulfone analyses were carried out according to Moore (235) as described in section 2.

(iii) Diagonal Paper Ionophoresis

Diagonal peptide maps were prepared by subjecting the peptic digest of the alkylated CHT-A₄ to initial ionophoresis at pH 6.5 and 50 volt/cm for 1 1/2 to 3 hours. The second dimension ionophoresis at pH 6.5 and 50 volt/cm was carried out for 1 1/2 hours in all cases. Peptides were detected with the cadmium-ninhydrin dip reagent (236). Histidine containing peptides were detected with the Pauly reagent.

(b) Chemical and Structural Studies on the Inhibited CHT-A₄

(i) Amino Acid Analyses and Methionine Sulfone Analyses

Amino acid analyses of CHT-CA, -ACK, -BCK and -βPECK are presented in Table XIII. The data indicate that as the number of methylene bridges between the aromatic and the chloromethyl ketone groups of the reagents increases, the alkylation of histidine residues also increased. Thus, CA and ACK do not alter the histidine content of CHT-A₄ whereas BCK and βPECK lead to a loss of 0.2 and 0.4 histidine residues respectively. The apparent recovery of methionine appears to follow a trend opposite to that observed for histidine, that is, as the number of methylene bridges increases, the extent of methionine alkylation decreases.

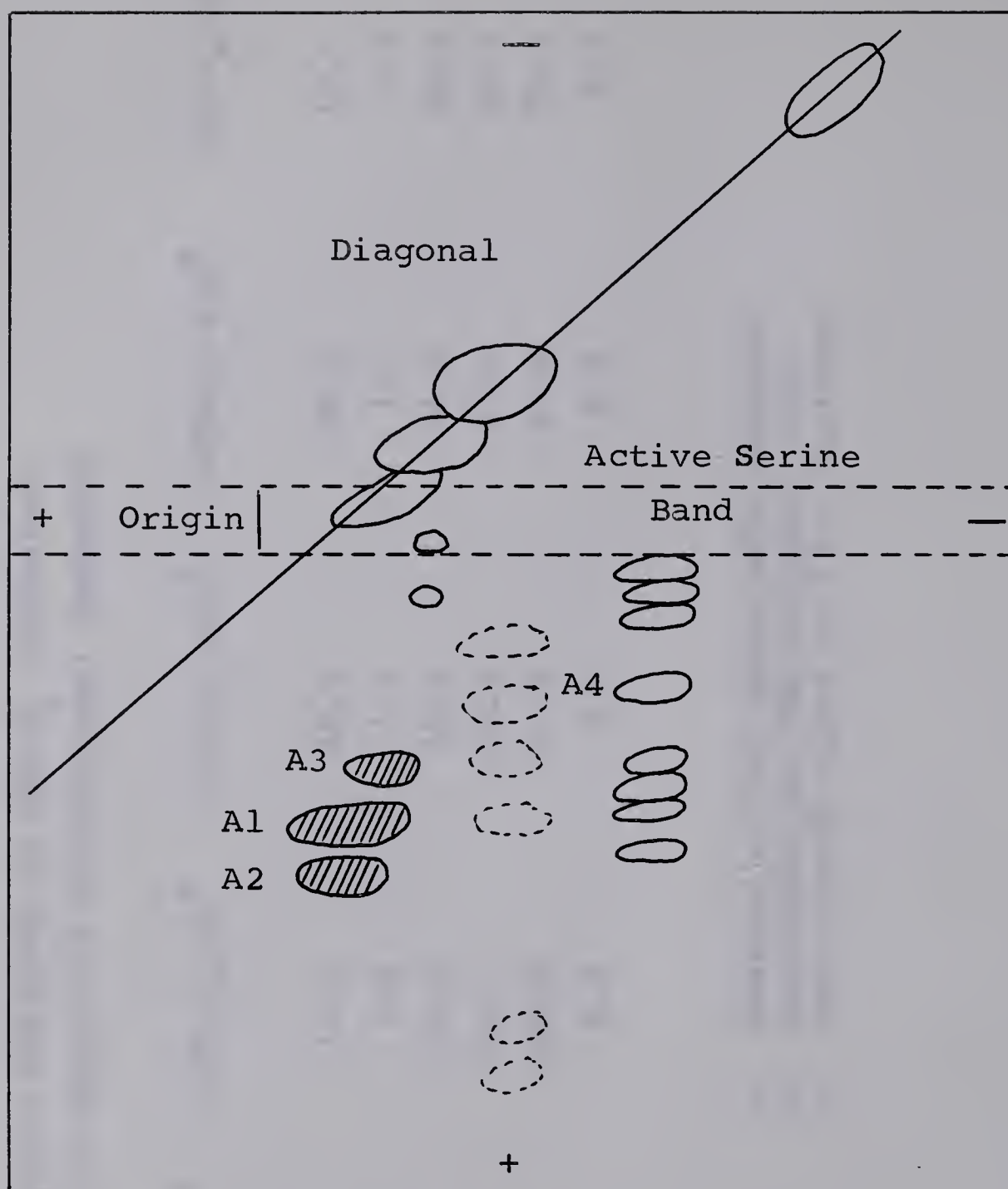
The presence of homoserine lactone on the amino acid chromatograms of CHT-A₄-CA and CHT-A₄-ACK was indicative of

TABLE XIV

Methionine Sulfone Analyses of CHT-A₄ Inhibited
with a Homologous Series of Bifunctional Reagents.

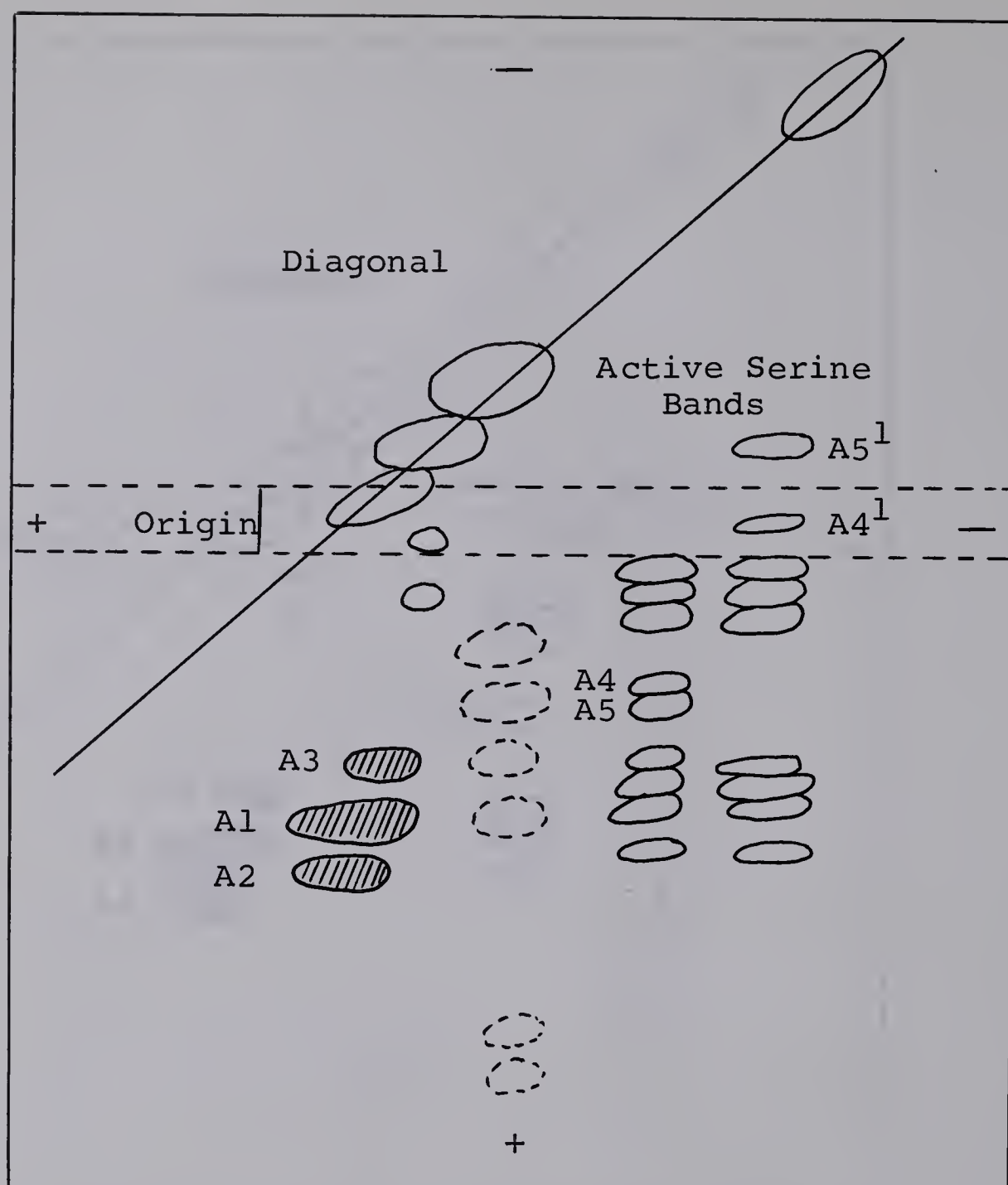
Amino Acid	CHT-A ₄ ^a	CHT-A ₄ -CA ^a	CHT-A ₄ -ACK ^a	CHT-A ₄ -BCK ^a	CHT-A ₄ -βPECK ^a
Cysteic Acid ^b	10.3	10.3	10.5	10.1	10.0
Methionine Sulfone	1.9	1.0	1.1	1.7	1.6
Glutamic Acid	14.7	14.6	15.0	14.8	14.8
Glycine	22.5	22.7	22.6	22.5	22.3
Alanine ^c	22.0	22.0	22.0	22.0	22.0
Methionine	-	0.2	0.2	0.1	0.1

- a. Average of two amino acid analyses
b. Corrected for 94% recovery (235)
c. Arbitrarily taken as 22.0 residues.



A1 is histidine-40 peptide
 A2 is histidine-57 peptide
 A3 is histidine-57 peptide
 with N-terminal threonine.
 A4 is methionine-192 peptide

Figure 35. Diagonal peptide map of the peptic digest of native CHT-A₄. Ionophoresis was run at pH 6.5⁴ and 50 volts/cm for 1.5 hours in both dimensions. Pauly positive, histidine containing peptides are hatched.



A1 is histidine-40 peptide
 A2 is histidine-57 peptide
 A3 is histidine-57 peptide with
 N-terminal threonine.
 A4 and A5 are methionine-192 peptides
 A4¹ and A5¹ are alkylated methionine-192
 peptides.

Figure 36. Diagonal peptide map of the peptic digest of CHT-A₄-CA and CHT-A₄-ACK. Ionophoresis was run at pH 6.5 and 50 volts/cm for 1.5 hours in both dimensions. Pauly positive, histidine containing peptides are hatched.

the presence of methionine sulfonium salts. Gundlach et al. (229) found that during acid hydrolysis, methionine carboxymethyl-sulfonium iodide decomposed to regenerate methionine and yield as one of the breakdown products homoserine lactone. It appeared then that the recovery of methionine from the alkylated CHT-A₄, as shown in Table XIII, was not a true measure of the free methionine content. Methionine was therefore determined as methionine sulfone following performic acid oxidation according to the method of Moore (235). The results of amino acid analyses are reported in Table XIV.

The analyses indicate that approximately one residue of methionine is transformed into its sulfonium salt when CHT-A₄ was inhibited with CA and ACK. These findings are in agreement with the studies of Schramm and Lawson (48) using α -bromoacetophenone.

Inhibition of CHT-A₄ with BCK and β PECK was accompanied by the loss of sub-molar amounts of methionine (0.3 and 0.4 residues, respectively). These reagents appear to exert their inhibitory effect through a combination of methionine and histidine alkylation. It is surprising that β PECK, which possesses the backbone structure of TPCK, does not lead to a greater loss of histidine.

(ii) Diagonal Peptide Maps of Native and Inhibited CHT-A₄

The diagonal peptide maps of the peptic digest of native CHT-A₄ and CHT-A₄ inhibited with CA and ACK are presented in Figures 35 and 36 respectively. In agreement with amino acid

analyses which indicated complete recovery of histidine from CHT-A₄-CA and CHT-A₄-ACK (Table XIII), no alteration in the histidine peptides was observed.

As a consequence of the extended ionophoresis periods, the Pauly positive histidine peptide A3 was observed. Amino acid analysis of the peptide was consistent with the histidine-57 peptide (A2) except that an additional threonine residue was present. The sequence immediately preceding the histidine-57 is known (11) to be VAL-VAL-THR-ALA-ALA-HIS-CYS- and is split by pepsin between the THR-ALA bond. However, Smillie and Hartley (16) have shown that the histidine-57 peptide from elastase possesses the sequence THR-ALA-ALA-HIS-CYS-VAL-ASP-ARG-GLX-. Since threonine-54 is the only residue of its type in the vicinity of the histidine-57 peptide in the sequence of CHT-A₄, it appears that a minor peptic split has occurred between VAL-THR. The slight increase in the mobility of the dihistidine-cystine peptide in the first dimension is likely a result of a small increase in the pK_a of histidine-57. A reduction in the field effect of the amino group of alanine on histidine-57 would be anticipated if the positive charge was removed by one residue to the N-terminal threonine. The marked difference in the mobilities of histidine-57 peptides A2 and A3 reflects the increase in molecular weight of the peptide (~10%) contributed by the threonine residue. A field effect exerted by the N-terminal threonine residue on the pK_a of histidine-57 would be nullified by the dominant field effect of the adjacent cysteic acid residue.

Methionine sulfone analyses indicated the presence of a residue of methionine sulfonium salt in CHT-A₄-CA and CHT-A₄-ACK. Two bands are observed in the active serine region of the diagonal peptide map in Figure 36. Of the two methionine residues present in CHT-A₄, only methionine-192 is found in this region (132). The increased mobility of the more basic band appears to be due to the presence of an additional positive charge contributed by the sulfonium salt of methionine-192. The slower band appeared to be derived from the faster band as a result of the decomposition of the sulfonium salt during ionophoresis and peptic digestion, with concomitant loss of the positive charge.

In an attempt to gain a more thorough understanding of the degradation of the methionine sulfonium salt formed by CA ($\text{C}_6\text{H}_5 \cdot \text{CO} \cdot \text{CH}_2\text{Cl}$), the sulfonium salt was synthesized according to the method of Gundlach et al. (255). α -chloroacetophenone (120 mg) and L-methionine (48 mg) (molar ratio: CA/Met = 2.5/1) were dissolved in 100 ml of 20% ethanol and incubated at 37° for 24 hours. Excess CA was removed by extraction with ether and the residual solution was freeze-dried. The powder obtained was dissolved in an appropriate volume of water, spotted 10 cm from the anode on a sheet of Whatman 3 MM paper and subjected to ionophoresis at pH 1.8 and 70 volts/cm for 30 minutes. In addition to the prominent methionine band, a second major band was observed to run in the vicinity of alanine. This band was eluted with water, spotted on 3 MM paper and re-subjected to ionophoresis under the original

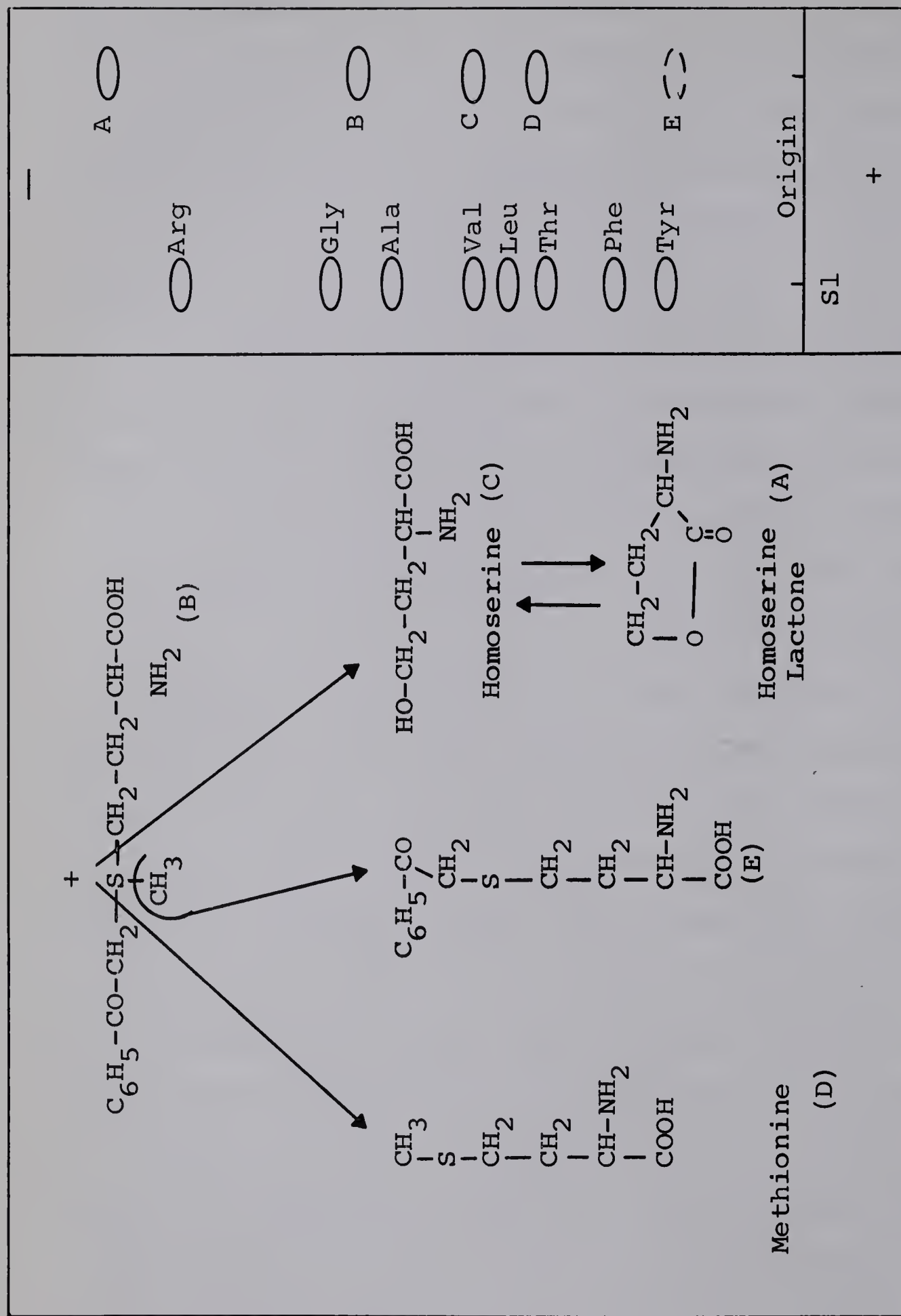
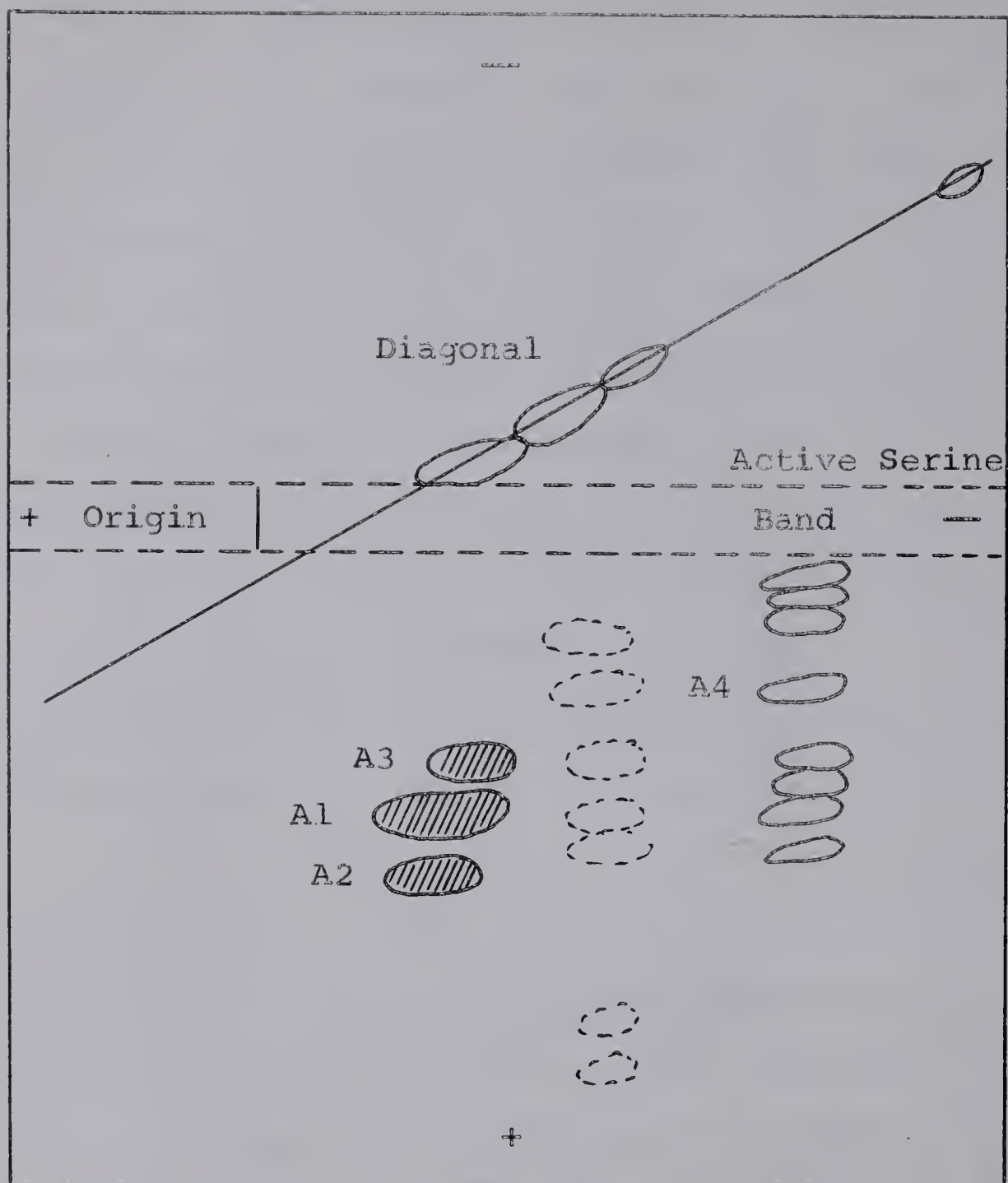


Figure 37. Proposed decomposition of the sulfonium salt formed from methionine and α -chloroacetophenone (left). Detection of the degradation products of the sulfonium salt on ionophoresis at pH 1.8 (Right).

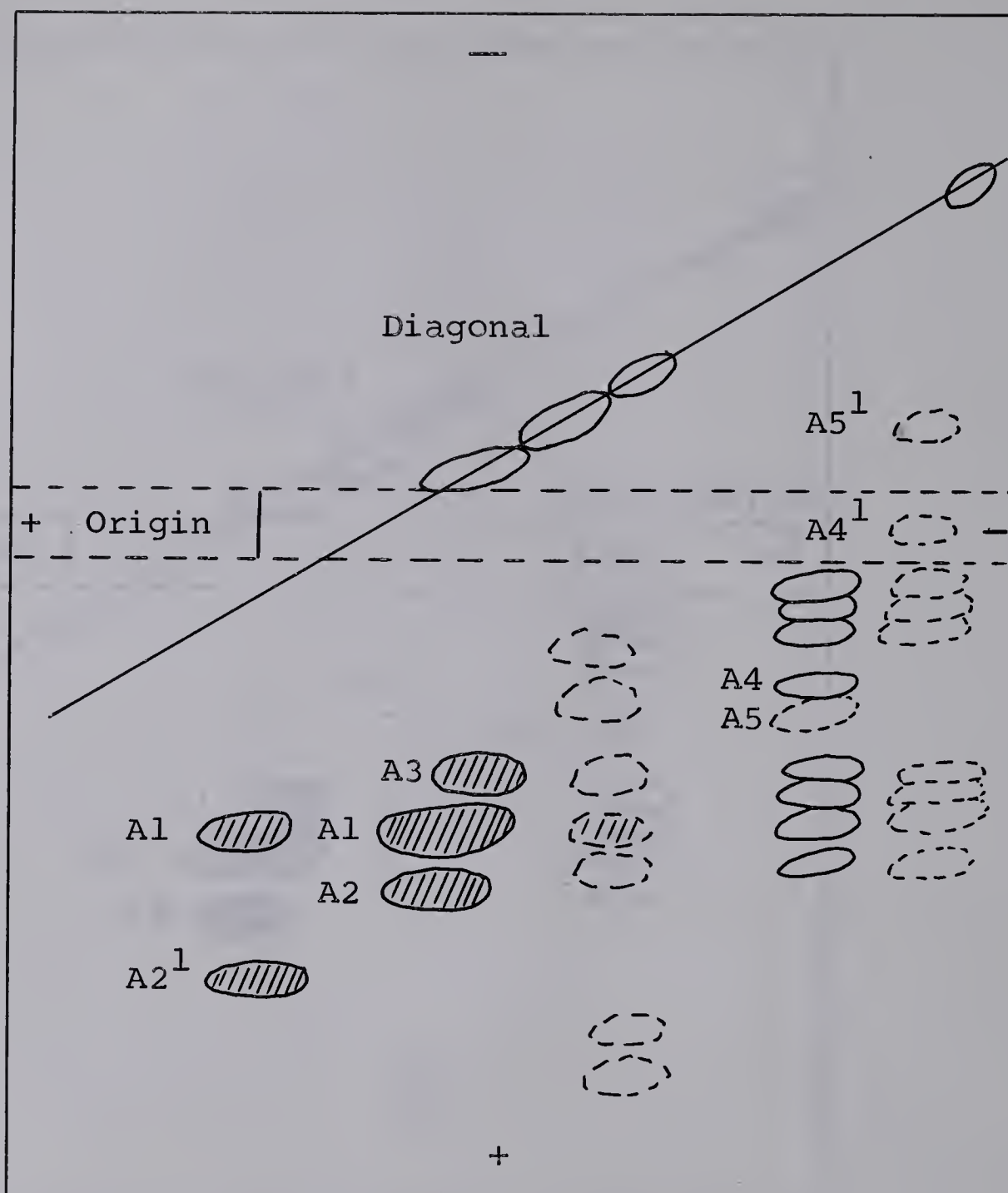
conditions. Following the elution of the "alanine" band with water, aliquots were removed and added to pH 1.8, pH 3.5 and pH 6.5 buffers. The solutions were incubated overnight at 37° and subjected to ionophoresis at pH 1.8 and 90 volts/cm for 15 minutes. Analysis of the degradation products and a proposed degradation pathway of the sulfonium salt is presented in Figure 37. Essentially identical degradation patterns were observed at the three pH values studied. Homoserine lactone stained yellow with the cadmium-ninhydrin reagent and was observed to emerge from the short column (11 cm) on the amino acid analyser just after ammonia. These model studies confirm the postulated transformation of the sulfonium salt peptides A4' and A5' into methionine containing peptides A4 and A5, respectively.

It should be noted that the active serine band on the diagonal peptide map of CHT-A₄ (Figure 35) is not completely analogous with the less basic active serine band on the diagonal peptide map of CHT-A₄-CA and CHT-A₄-ACK (Figure 36). Peptides A5 and A5¹ which stained red with the cadmium-ninhydrin reagent, are present in the latter, but are absent in the former peptide map. The orange (cadmium-ninhydrin) peptide A4 was present on both peptide maps, however, it appears to be noticeably less concentrated on the diagonal of CHT-A₄-CA and -ACK. A possible explanation for the appearance of peptides A5 and A5¹ could be the steric influence of the S-alkylated methionine residue on the cleavage of a susceptible peptide bond by pepsin. Thus, peptide A4 (or A4¹) was derived from peptide A5 (or A5¹) by partial cleavage of a peptide bond by pepsin on the alkylated



A1 is histidine-40 peptide
 A2 is histidine-57 peptide
 A3 is histidine-57 peptide with
 N-terminal threonine
 A4 is methionine-192 peptide

Figure 38. Diagonal peptide map of the peptic digest of native CHT-A₄. Ionophoresis was run at pH 6.5 and 50 volts/cm. First dimension: 3 hours. Second dimension: 1.5 hours. Pauly positive, histidine containing peptides are hatched.



A1 is histidine-40 peptide
 A2 is histidine-57 peptide
 A2¹ is alkylated histidine-57 peptide
 A3 is histidine-57 peptide with N-terminal threonine.
 A4 and A5 are methionine-192 peptides
 A4¹ and A5¹ are alkylated methionine-192 peptides.

Figure 39. Diagonal peptide map of the peptide digest of CHT-A₄-BCK and CHT-A₄-PECK. Ionophoresis was run at pH 6.5 and 50 volts/cm. First dimension: 3 hours. Second dimension: 1.5 hours. Pauly positive, histidine containing peptides are hatched.

CHT-A₄. In native CHT-A₄, peptide A5 was not observed since peptide A4 was liberated completely by pepsin.

The diagonal peptide maps of the peptic digests of CHT-A₄ and CHT-A₄ inhibited with BCK and βPECK are presented in Figures 38 and 39 respectively. Ionophoresis conducted for 3 hours in the first dimension and 1 1/2 hours in the second dimension resulted in an excellent separation of histidine and the active serine band. The diagonal peptide maps of the peptic digests of CHT-A₄-BCK and CHT-A₄-βPECK were qualitatively identical. However, the intensity of the Pauly and ninhydrin colors for the paired A1 and A2 peptides was noticeably less in CHT-A₄-BCK than in the case of CHT-A₄-βPECK. This observation is in agreement with the amino acid analyses which indicated that 0.2 and 0.4 residues of histidine were alkylated with BCK and βPECK respectively.

Two intriguing features of the diagonal peptide map (Figure 39) present themselves. Firstly, peptide A2¹ which appears to be on alkylated histidine-57 peptide, is Pauly positive. (It will be recalled that 3-CM histidine-57 peptide from CHT-TPCK and -PMCK was Pauly negative). Secondly, a trace of histidine-40 peptide A1 exists to the right of the cluster of native histidine peptides (A1, A2 and A3). The mate of this histidine-40 peptide was not discernable. The position of the trace amounts of peptide A1 on the diagonal indicate that its precursor dihistidine cystine peptide must have been more basic than the native dihistidine cystine peptide which yielded peptides A1, A2 and A3 after performic

acid oxidation. Since this basic band was observed only on the diagonals of CHT-A₄-BCK and -βPECK, it appears to be an unique and to date, unknown, product of the alkylation. Several attempts to isolate the histidine-40 and histidine-57 peptides in this region were without success.

The isolation and characterization of peptides A1 and A2¹, which originated from a slightly anionic dihistidine cystine peptide remaining at the origin after the first dimensional ionophoresis, are presented in the following section.

The presence of a dominant (orange) A4 peptide on the diagonal peptide map of CHT-A₄-BCK and -βPECK conforms to the methionine sulfone analyses which indicated that 1.6 residues of free methionine were present. On the basis of earlier studies on CHT-A₄-CA, it appears that 0.6 residues of methionine-192 are not alkylated in CHT-A₄-βPECK. Thus, peptic hydrolysis of CHT-A₄-βPECK (or -BCK) would be expected to yield substantial amounts of peptide A4 as was shown to be present on the diagonal of native CHT-A₄. The existence of 0.4 residues of methionine sulfonium salt in the preparation would be expected to produce peptide A5 and A5¹ in lower concentrations than were observed with CHT-A₄-CA.

(iii) Isolation and Characterization of Methionine and Histidine Peptides.

Brown and Hartley (132) have shown that the methionine-192 was present in a cysteic acid peptide in the active serine band on the diagonal peptide map of CHT-A₄. The particular peptide was designated 1C1 and was composed of residues 190 to 207.

Furthermore, the cadmium-ninhydrin color of this peptide (N-terminal serine) was distinctively orange. Since an orange staining peptide was obtained in the active serine band on the diagonal peptide maps of CHT-A₄ (Figures 35 and 38) and since this particular peptide possessed an altered mobility in the basic (sulfonium salt) active serine band, it was reasonable to assume that peptide A4 corresponded to the methionine containing peptide (1C1) isolated by Brown and Hartley (132).

Attention was directed to determining the nature of peptides A5 and A5¹ (Figure 36) which stained red with cadmium-ninhydrin and appeared to contain methionine since the mobility of peptide A5¹ was altered in the basic active serine band. A pepsin digest of CHT-A₄-CA (55 mg) was spotted as a 6 x 40 cm band 20 cm from the anode on a Whatman 3MM paper and subjected to ionophoresis at pH 6.5 and 50 volts/cm for two hours. The positions of the basic and regenerated active serine bands were determined from a diagonal peptide map. The active serine bands were cut out, oxidized, sewn 23 cm from the anode on sheets of Whatman 3MM paper and subjected to ionophoresis at pH 6.5 and 50 volt/cm for 2 1/2 hours. The neutral region, containing peptide A5¹, was cut out from the basic active serine band, eluted with water and reappplied a distance of 10 cm from the anode on a sheet of What #1 paper. Purification of the sulfonium salt peptide was obtained by subjecting the neutral region to ionophoresis at pH 1.8 and 70 volts/cm for thirty minutes. Streaking was evident behind the major peptide band running near CM-cysteine. Following

TABLE XV

Amino Acid Analyses of Methionine Peptides from
 CHT-A₄-CA

Amino Acid	A5 ^a	Peptide A5 ¹ ^a	A5 ^b	1C1 ^c
Lysine	1.7	2.0	2	1.8
Cysteic Acid	1.9	1.5	2	1.8
Methionine Sulfone	0.9	-	-	0.9
Aspartic Acid	2.0	1.8	2	2.0
Threonine	0.2	0.2	-	0.5
Serine	2.6	2.5	3	2.1
Proline	0.7	0.5	1	1.1
Glycine	4.7	4.4	4	4.1
Alanine	1.0	1.3	1	1.0
Valine	2.0	1.6	2	1.0
Methionine	-	0.2	1	-
Leucine	1.0	1.0	1	1.0

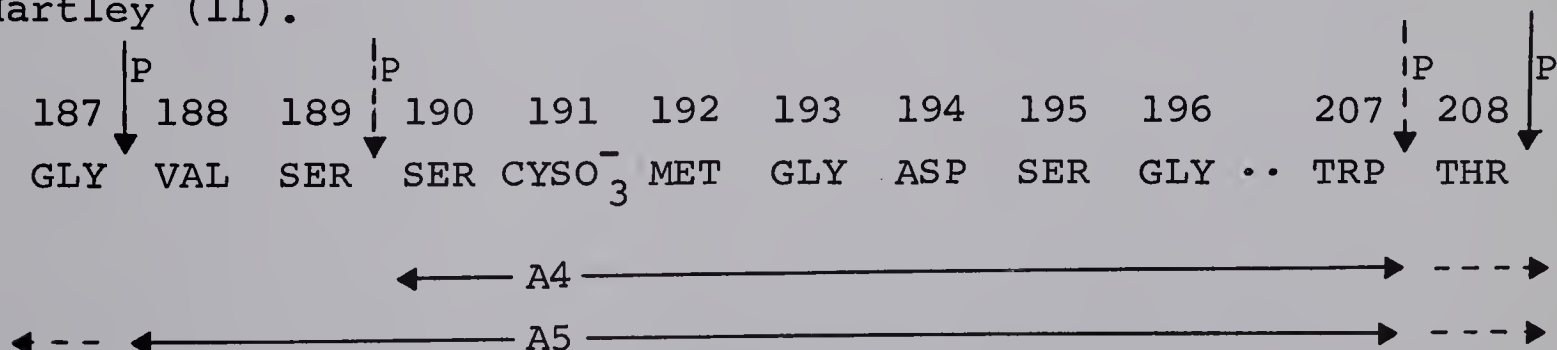
- a. See Figure 36.
- b. Theoretical values based on amino acid sequence of CHT-A₄(11).
- c. Data of Brown and Hartley (132) corresponds to peptide A4 in this study.

elution from the paper with water, an equal volume of concentrated HCl was added and the peptide was hydrolyzed as outlined earlier. Amino acid analysis of A5¹ is presented in Table XV.

Peptide A5 was eluted from the regenerated active serine band with water, reapplied to Whatman #1 paper and was subjected to ionophoresis at pH 1.8 and 70 volt/cm for thirty minutes. The peptide migrated to a point approximately 9 cm from the origin. Amino acid analysis is presented in Table XV.

The absence of methionine sulfone and presence of a trace of methionine in the analysis of peptide A5¹ strongly suggest the presence of a methionine sulfonium salt. Furthermore, the recovery of methionine sulfone from A5 and the favourable agreement between the analyses of A5 and A5¹ substantiate the proposal that peptide A5 was derived from peptide A5¹ via decomposition of the sulfonium salt of methionine-192.

The structure of peptides A4 and A5 was deduced from amino acid analyses and cadmium-ninhydrin color reactions in conjunction with the work of Brown and Hartley (132) and Hartley (11).



Some uncertainty exists as to whether or not glycine-187 should be included in peptide A5. Since glycine peptides are usually yellow in color and peptide A5 (and A5¹) stained red with the cadmium-ninhydrin, it appears more likely that valine is the N-terminal residue.

TABLE XVI

Amino Acid Analyses of Histidine Peptides from
 CHT-A₄-βPECK

Histidine-40 Peptides

Amino Acid	A1 (Paired to A2 ¹)	A1 (Paired to A2)	Smillie <u>et al.</u> (16)
Histidine	0.7	0.6	1
Cysteic Acid	1.0	1.1	1
Serine	1.0	1.0	1
Glycine	2.0 ^a	2.0 ^a	2
Leucine	1.0	1.0	1
Phenylalanine	0.9	0.9	1

Histidine-57 Peptides

Amino Acid	A2 ¹	A2	Smillie <u>et al.</u> (16)
Histidine	-	1.0	1
Cysteic Acid	0.9	0.9	1
Aspartic Acid	1.0	1.0	1
Threonine	1.8	1.9	2
Serine	1.0	0.9	1
Glycine	1.0	1.0	1
Alanine	2.0 ^a	2.0 ^a	2
Valine	0.9	1.0	1

a. Arbitrarily taken as 2.0 residues.

Due to the limited amount of peptide A5 and A5¹ on the diagonal peptide maps of CHT-A₄-BCK and -βPECK, no attempt was made to isolate these peptides. The similarity in the active serine region of Figures 36 and 39 strongly suggested that the modification of methionine was analogous.

The purification of the histidine peptides (A1, A2 and A2¹) from CHT-A₄-βPECK was achieved in a similar manner as described in section 2 iii. Interestingly, the mobility of the Pauly positive peptide A2¹ was identical to the native histidine-57 peptide (A2) during ionophoresis at pH 3.5 ionogram, hydrolysed with HCl and a portion of the hydrolysate was subjected to quantitative amino acid analyses (see Table XVI).

Histidine-40 peptides (A1) were identical as was suggested from their observed mobilities on the diagonal peptide map (Figure 39). However, the Pauly positive peptide A2¹ was identical to the native histidine-57 peptide except that histidine could not be detected.

Qualitative amino acid analysis of peptide A2¹ was conducted on Whatman #1 paper by first subjecting the acid hydrolysate, in parallel with appropriate standard amino acids, to ionophoresis at pH 6.5 and 50 volt/cm for fifty minutes. The neutral band was removed, sewn on to a second sheet of Whatman #1 paper and subjected to ionophoresis at pH 1.8 and 70 volt/cm for 40 minutes. Separate portions of the pH 6.5 and pH 1.8 ionograms were developed with cadmium-ninhydrin reagent and the Pauly reagent. Histidine or a Pauly positive

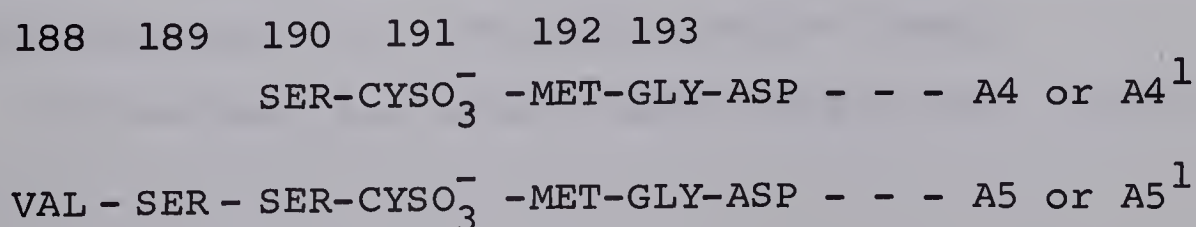
derivative was not present on the pH 6.5 ionogram. However, a new ninhydrin positive amino acid was observed migrating behind alanine on the pH 1.8 ionogram of the neutral band from pH 6.5.

c. Discussion

The studies presented indicate that a methionine residue in CHT-A₄ is alkylated by CA and ACK whereas only a partial alkylation of methionine occurs with BCK and βPECK. Diagonal peptide maps of the native and alkylated CHT-A₄ and amino acid analysis of an isolated methionine sulfonium salt peptide confirm the alkylation of methionine-192. Evidence from the diagonal peptide maps suggests that methionine-180 is not modified.

An apparent anomaly exists in Figure 36 when the mobilities of peptides A4 and A5 are related to the number of amino acid residues in the peptides. Previous discussions have indicated that peptide A5 is larger than peptide A4 and thus would be expected to migrate behind A4 rather than in front of it as observed in Figure 36. Furthermore, it appears somewhat surprising that peptides A4¹ and A5¹ possess such altered mobilities relative to each other following the formation of a sulfonium salt with a common reagent.

A consideration of the net charges of the peptides offers a reasonable explanation of the irregularities. Attention is drawn to the N-terminal sequences of peptides A4 and A5.



Through the influence of the field effect of the negatively charged cysteic acid residue, the pKa of the α -amino group of the N-terminal serine residue in peptide A4 would be increased. Concomitant with this increase would be an increase in the positive charge. The net effect would be to decrease the net negative charge on the peptide thus leading to a retardation in its anionic migration at pH 6.5. However, the α -amino group of N-terminal valine in peptide A5 would only be weakly influenced by the cysteic acid residue, and as such, would possess less positive charge than the corresponding group in peptide A4. Thus an approximation of the net charge on peptides A4 and A5 indicates -1 and -1 1/4 charges respectively. This charge difference appears to be suffice to cause the larger peptide A5 to move just ahead of the smaller peptide A4.

The mobility of peptides A4¹ and A5¹ may also be explained on a basis of the field effects exerted by cysteic acid -191. With the presence of a positively charged methionine sulfonium salt adjacent to cysteic acid -191, the field effect of the latter residue on serine -190 could conceivably be partially nullified and lead to a slight drop in the pKa of the α -amino group of N-terminal serine in peptide A4. The net charge on peptides A4¹ and A5¹ is estimated to be approximately the same (δ^-) on both peptides. Thus the positions of A4¹ and A5¹ in Figure 36 appear to be in accordance with a separation based on a molecular weight basis.

It has been previously demonstrated that during performic

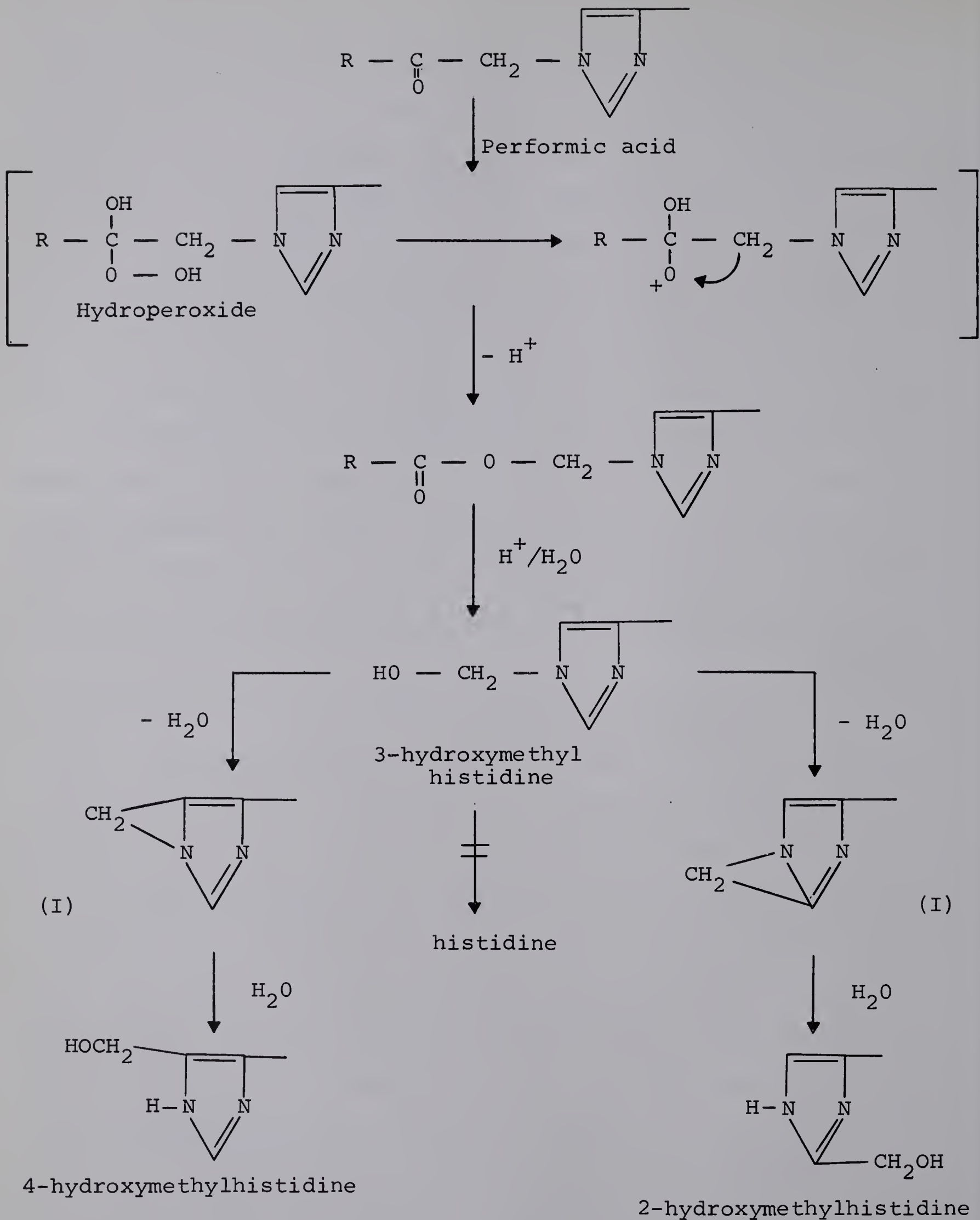


Figure 40.

A plausible mechanism for the performic acid rearrangement of BCK or βPECK histidine derivatives to yield 2 (or 4)-hydroxymethylhistidine.

acid oxidation a rearrangement of the ketone moiety of TPCK-histidine and PMCK-histidine led to the formation of the 3-carboxymethyl derivative of histidine. The groups migrating in the above reaction were the L-1-tosylamido-2-phenylethyl and phenoxymethyl moieties. A similar rearrangement does not appear to occur in the β PECK-histidine derivative. Unlike the side chains of TPCK and PMCK, β PECK does not possess electronegative atoms in proximity to the ketone group. This structural difference probably leads to an altered peracid rearrangement.

Cleavage of the C-C bond adjacent to the ketone group can occur either to the "left" or to the "right" depending on the migratory aptitude of the various R groups.

The hypothetical pathway outlined in Figure 40 suggests that performic acid rearrangement of an assumed nitrogen 3 alkylated imidazole derivative occurs at the C-C bond to the "right" of the ketone group. The 3-hydroxymethylhistidine would not be expected to be stable and would either decompose to yield free histidine and formaldehyde, or rearrange to a more stable entity such as 2- or 4-hydroxymethylhistidine. Support for the proposed rearrangement includes: 1. three membered ring structures, as envisaged with intermediate I (Figure 40) are known to exist and are labile to acid medium (for example; ethylenimine $\text{CH}_2\text{--}\overset{\text{H}}{\underset{\text{H}}{\text{N}}}\text{--CH}_2$ and 2. the presence of native histidine-57 peptide (A2), which could arise through decomposition of a 3-HM histidine peptide, was not observed between peptide A1 and A2¹ on the diagonal peptide map (Figure 39).

The proposed N-alkylated histidine-57 peptide and the peracid induced rearranged derivative-2-(or 4) hydroxymethyl-histidine-are consistent with the available data. Inductive effects (-I) of the β PECK substituent on the histidine-57 residue decrease the pKa of the imidazole group which leads to a reduction in the net positive charge on the dihistidine cystine peptide, thereby causing the peptide to remain at the origin on the initial pH 6.5 ionophoresis. Following performic acid oxidation, histidine-40 peptide and the proposed 2- (or 4) hydroxymethylhistidine-57 peptide (2-(or 4) HM histidine) (A2¹) would be present. It has been shown (Table IX) that the net charge on native histidine-57 peptide is approximately -1.2 at pH 6.5 (based on a pKa \sim 7.2 on imidazole group). Hofmann (126) has indicated that a 4-hydroxymethyl substituent on imidazole decreases the pKa by 0.6 units. In addition, a substituent introduced into the 2-position is known to have a more pronounced inductive effect than the same substituent at the 4-position (for example; imidazole, pKa 6.95; 2-methylimidazole, pKa 7.86; 4-methylimidazole, pKa 7.52). Thus, a 2-hydroxymethyl derivative of histidine-57 could depress the pKa of the imidazole group about 0.8 units and lead to a net charge of -1.5. This value appears to be in accord with the observed mobility of peptide A2¹ relative to peptide A2 (Figure 39). Clearly, the deductions presented which infer the 2-hydroxymethyl derivative are speculative and do not eliminate a 4-hydroxymethylhistidine.

The identical mobilities of peptide A2 and A2¹ at pH 3.5

are in agreement with the postulated 2 (or 4)-HM histidine-57. Moreover, the isolation of a neutral histidine derivative at pH 6.5 from the acid hydrolysate of A2¹ is also consistent. A 2- or 4- hydroxymethyl group could depress the pKa of the imidazole group of free histidine (pKa = 6.1) at least 0.5 units and thereby form an uncharged imidazole at pH 6.5. If a linear relationship exists between net charge and molecular weight, then 2 (or 4)- HM histidine (M.W. 186, net charge + 1.5) would be expected to migrate behind alanine (M.W. 89, net charge + 0.8) during ionophoresis at pH 1.8.

The data presented herein indicate that β PECK alkylates histidine-57 of CHT-A₄. Similarities in the diagonal peptide map of CHT-A₄-BCK and CHT-A₄- β PECK suggest that BCK also alkylates this residue of CHT-A₄. However, the position of alkylation on the imidazole has only been tentatively assigned to the nitrogen 3 position.

The homologous series of phenylalkyl chloromethyl ketones presently studied ($C_6H_5 \cdot [CH_2]_n - CO \cdot CH_2Cl$ where $n = 0, 1$ and 2) inhibit CHT-A₄ in a manner not analogous to the phenylalkylamido bromomethyl ketone series ($C_6H_5 \cdot [CH_2]_n \cdot NH \cdot CO \cdot CH_2Br$) of Schramm and Lawson (48). These workers also studied benzyl bromide ($C_6H_5 \cdot CH_2Br$) and α -bromoacetophenone ($C_6H_5 \cdot CO \cdot CH_2Br$). With the exception of the $n = 1$ compound, they observed that the closer the bromine atom to the benzene ring the more rapid is the inactivation of CHT-A₄. Only a methionine residue, assumed to be residue 192, was modified by these reagents. Residual activity of the alkylated CHT-A₄ was determined to be less than 7% as assayed against TEE. The reduction in activity was shown

to be associated with an increase in the K_m of the TEE and was supported by the stoichiometric incorporation of DFP into the modified enzymes.

Since CA ($C_6H_5 \cdot CO \cdot CH_2Cl$) and ACK ($CH_3O \cdot C_6H_4 \cdot CO \cdot CH_2Cl$) inhibit CHT- A_4 by virtue of the S-alkylation of methionine-192, it may be concluded that the observed residual activity of 15% and 20% respectively (assayed against ATEE) reflects a reduction in enzyme affinity for ATEE. The apparent discrepancy in the residual activity of CHT- A_4 inhibited with CA and α -bromoacetophenone (20% and 2% respectively) likely arises from the assay methods employed. The protonated α -amino group of TEE would be expected to be repelled by the positively charged sulfonium salt and thus would lead to a lower residual activity than if the assays were performed with ATEE.

Comparison of the two series of bifunctional reagents reveals that two out of the three compounds in each series are remarkably similar in structure. Thus, the following compounds of Schramm and Lawson (48) $C_6H_5 \cdot NH \cdot CO \cdot CH_2Br$ and $C_6H_5 \cdot CH_2 \cdot NH \cdot CO \cdot CH_2Br$ resemble $C_6H_5 \cdot CH_2 \cdot CO \cdot CH_2Cl$ (BCK) and $C_6H_5 \cdot CH_2CH_2 \cdot CO \cdot CH_2Cl$ (β PECK) employed in the present study. Since a methylene bridge does not possess the hydrogen bond forming capabilities, nor the electronegative nature of the -NH-group, it appears possible that the differences in the modes of inhibition of CHT- A_4 by the reagents could stem from differences in the binding of the reagents to the active center. The phenylalkylamido moiety ($C_6H_5 \cdot [CH_2]_n \cdot NH-$) may well bind preferentially to the acylamido binding site of CHT- A_4 through a combination of hydrogen and hydrophobic bonding.

Once bound in this position, the reactive bromomethyl ketone would be in proximity to methionine-192 which is known to be situated near the binding site (88, 91). BCK and β PECK, on the other hand, would likely be preferentially bound to the specific binding site. Such a possibility appears to have merit since molecular models of $C_6H_5 \cdot CH_2 \cdot NH \cdot CO \cdot CH_2Br$ and $C_6H_5 \cdot CH_2CH_2 \cdot CO \cdot CH_2Cl$ reveal that the distances between the aromatic and halomethyl ketones are almost identical.

The correlation between percent residual activity and the extent of histidine and methionine alkylation in CHT-A₄-BCK and CHT-A₄- β PECK presents a perplexing problem. Amino acid and methionine sulfone analyses of CHT-A₄ inhibited with BCK and β PECK indicate that 0.2 and 0.4 residues of histidine and 0.3 and 0.4 residues of methionine are alkylated. Histidine-57 alkylation yields a completely inactivated enzyme whereas methionine-192 alkylation results in a variable amount of residual activity, depending on the size of the substituent. If it is assumed, for the sake of this discussion, that a methionine modification essentially yields an inactivated enzyme, then the sum of the histidine and methionine residues alkylated should correlate with loss of activity. The net effect of these alkylations would be expected to yield 50% residual activity in CHT-A₄-BCK and 20% residual activity in CHT-A₄- β PECK. Clearly, these estimates reflect the maximum amount of residual activity possible provided that the histidine and methionine alkylations are mutually exclusive.

Assays of the residual activity of CHT-A₄-BCK and

CHT-A₄-βPECK indicated that the percent residual activity was 35% and 3% respectively. It would appear that some additional residue is being modified and contributing to the observed inhibition. A candidate for the residue involved would be the active serine-195. Since studies conducted with CHT-A₄-TPCK and -PMCK revealed that histidine and methionine alkylation were not mutually exclusive, it would appear that a similar situation could exist in CHT-A₄-BCK and CHT-A₄-βPECK. Thus, alkylation of the active serine residue could be appreciable. The solution to this problem appears to reside in the use of a labelled inhibitor (βPECK) coupled with the diagonal technique.

Although the alkylation of serine-195 by BCK and βPECK has been postulated to contribute to the inhibition of CHT-A₄, other residues could conceivably be involved. In particular, the α-amino group of isoleucine-16 is an attractive candidate. The alkylation of the indole ring of tryptophan, the phenolic group of tyrosine and the ε-amino group of lysine could possibly be modified by the reagents. Furthermore, the formation of an ester linkage with aspartic-194, adjacent to the active serine, is feasible.

4. Conclusions

From the studies presented in this thesis and in the literature, certain conclusions regarding the requirements for alkylation of histidine-57 and methionine-192 in CHT-A₄ may be drawn. Alkylation of histidine-57 by bifunctional reagents appears to be quite restrictive and depends on the presence

of a chloromethyl ketone group and on the exact spatial relationship between the chloromethyl ketone and the aromatic moiety. The precise spatial relationship is attained by the presence of two methylene bridges (L-TPCK, β PECK) or a combination of an ether linkage and a methylene bridge (PMCK). Methionine alkylation, on the other hand, appears to be non-specific and occurs readily with a variety of reagents differing not only in the nature of the alkylating group but also in the distance separating the aromatic and the alkylating group.

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Appendix A

Ionophoresis

High-voltage ionophoresis was performed in the vertical strip apparatus of Michl (213, 214) as modified by Ryle et al. (256). For a definitive discussion of this technique the reader is referred to an excellent monograph by J. Leggett Bailey (257).

The apparatus used in the present studies was constructed from an all-glass Shandon Chromatank (22 1/2" x 9" x 22 1/2" deep) purchased from Consolidated Laboratories (Canada) Ltd.

Stainless steel electrode connectors were mounted in holes bored below the trough supports and were made leak-proof with teflon washers. Platinum electrodes were used exclusively. Constant voltages up to 5 Kv (D.C.) were maintained by Savant high-voltage instruments.

The upper glass buffer trough (cathode) was held in position by a glass rack fitted to the integral trough supports. Approximately two inches of buffer in the bottom of the tanks served as the anode solution. Suitably shaped glass rods prevented the paper from touching the sides of the vessel.

Lucite lids supported glass cooling coils and were connected to an interlock safety system. Lucite doors, also equipped with interlocks, were extremely useful since the paper ionophoresis could be viewed while in operation. Due to the toxic nature of the coolants (toluene/pyridine), the units were placed in a fume hood.

The buffers and coolants used in the ionophoresis tanks were as follows:

<u>pH 1.8</u>	
Buffer:	2% formic acid (Fisher certified reagents)
(207)	8% acetic acid (Fisher certified reagents)
Coolant:	Varsol (Imperial Oil Co.)
<u>pH 3.5</u>	
Buffer:	1890 ml deionized water
(207)	10 ml pyridine (Fisher certified reagent)
	100 ml glacial acetic acid
Coolant:	Varsol
<u>pH 6.5</u>	
Buffer:	879 ml deionized water
	100 ml pyridine
	3 ml glacial acetic acid
Coolant:	92% toluene (Fisher certified reagent)
	8% pyridine
	(by volume)

Standard amino acid mixtures were conveniently prepared by combining 200 μ l of a stock solution of each amino acid (0.001 M) as indicated below:

- S1 CM-CYS, TYR, PHE, THR, LEU, VAL, ALA, GLY, ARG, CYSTEIC
S2 METSO₂, ASP, GLU, PRO, ILE, SER, ALA, HIS, LYS, CYSTEIC

A 10 μ l aliquot of the amino acid mixtures (S1 and S2) contained 0.01 μ moles of each amino acid. On occasion it has been useful to double the concentration of aspartic acid and histidine in order to obtain clearly discernable spots.

When paper ionophoresis was conducted at pH 6.5 for extended periods of time (2 hours or more), it was imperative that the paper be thoroughly saturated with the buffer prior to ionophoresis in order to prevent excessive drying of the paper during the run. The use of the transparent lucite on the

compartment doors and tank lids enabled the operator to detect potential difficulties. Drying of the paper was accompanied by the formation of a translucent band across the paper.

Dansyl Amino Acid Standard Solutions:

S_D1 ALA, ARG, ASP, GLU, GLY, HIS, ILE, LEU, METSO₂, SER

S_D2 ARG, ASP, GLU, LYS, PHE, PRO, THR, VAL

Preparation

Stock amino acid solution were prepared by dissolving 65 μ moles of each amino acid in 10.0 ml of 0.1 M NaHCO₃. Into a series of centrifuge tubes was added 1.0 ml of a stock amino acid solution and 1.0 ml of Dansyl chloride (6.0 mg/ml in acetone). The mixtures were covered and allowed to stand overnight at 25°. NaHCO₃ was precipitated by adding 8 ml of acetone. Following centrifugation the supernatant was removed and stored at 3°.

Dansyl amino acid standard solution (S_D1 and S_D2) were prepared by mixing 2.0 ml of each stock Dansyl amino acid solution, evaporating to dryness, and dissolving the residue in 2.0 ml of acetone. A 2 - 3 μ l aliquot was spotted as standards.

Appendix B

Detection Sprays and Reagents

1. Cadmium-ninhydrin (Heilman et al. (236))

The reagent was prepared by adding 15 ml of a stock cadmium acetate solution (5g. cadmium acetate, (Fisher Certified Reagent), 250 ml glacial acetic acid and 500 ml water) to 100 ml of 1% (w/v) ninhydrin (Pierce Chemical Co.) in acetone (Fisher Reagent Grade). Papers were dipped into the solution and the colors were developed at 60° for 20 minutes. Depending on the nature of the N-terminal amino acid, various colors were produced. The following table is based upon empirical findings and is representative of the colors obtained with this useful reagent.

<u>Color</u>	<u>N-Terminal Amino Acid</u>
Yellow (stable 1 week) -----> RED	GLY, SER, THR, CYSH, PRO CYSTEIC
Orange	SER, HIS
Slow Red (overnight)	ILE, VAL
Fast Red	LEU, LYS, ARG, ASP, GLU, TYR, PHE, MET, METSO ₂ , TRP, ALA

2. Ordinary Ninhydrin

The reagent was 0.20 - 0.25% (w/v) ninhydrin in acetone and was used as a dip reagent. With the exception of proline, all amino acids stained purple after developing the color for 30 minutes at 60°.

3. Starch-Iodine Reagent for Peptides (258).

The paper was first sprayed with a 1/100 dilution of Javex (Domtar Consumer Products Ltd.) in water and allowed to dry at room temperature (10 minutes). Subsequent to spraying with ethanol (95%) and drying in an oven at 40°, a mixture of equal volumes of 1% potassium iodide and soluble starch (Nutritional Biochemicals Corp.) (final concentration of each 0.5%) was sprayed on to the paper. Peptides appeared as purple bands on a light grey background.

Compounds containing a -NH-group (peptides and acylated amino acids) are readily detected. The reaction appears to be



This detection reagent may be used after ninhydrin

4. Pauly Reagent for Histidine.

(Diazotized sulfanilic acid) (259)

a. Synthesis of the reagent:

Sodium carbonate (2g) and sulfanilic acid (2.2g, Fisher Certified Reagent) was dissolved in 50 ml of warm water. Sodium nitrite (2.2g, Fisher Certified Reagent) in 10 ml of water was added and the solution was cooled in an ice-bath. Hydrochloric acid (8 ml conc. HCl in 15 ml water) was slowly added with stirring over a period of 20 minutes. After precipitation of the reagent was completed, (1 hour) it was collected on a filter and stored as a paste in water at 3°.

b. Detection Spray:

A freshly prepared solution of diazotized sulfanilic acid (0.5 ml of paste in approximately 20 ml of cold 5% Na₂CO₃) was sprayed on to thoroughly dried papers. Histidine and

histidine peptides gave an orange-red spot (sensitivity 0.1 μ g). Tyrosine also reacted with the Pauly reagent to yield a pale orange spot which rapidly fades. On occasion, detection of histidine peptides on pH 1.8 ionograms not thoroughly dried was unsatisfactory. No difficulties were encountered with the Pauly reaction on pH 3.5 or 6.5 ionograms. Pauly positive spots were clearly visible even a year following the preparation of the ionogram.

c. Alternate Pauly for Histidine:

This reagent may be used after 0.5% ninhydrin in acetone. Equal volumes of 1% sulfanilic acid in 10% HCl and 45% NaNO_2 in water were mixed and allowed to stand at 0° for 15 minutes. The ionogram was subsequently sprayed with this reagent and allowed to dry at room temperature. Histidine peptides were detected as orange-red spots by spraying with a solution of 10% Na_2CO_3 .

5. Ehrlichs Reagent for Tryptophan.

Ionograms were dipped in a freshly prepared solution of 1% *p*-dimethylaminobenzaldehyde (Matheson, Coleman and Bell Co.) in 90 ml of acetone and 10 ml of concentrated HCl. In about 5 to 10 minutes, at room temperature, mauve to blue spots developed to indicate the presence of tryptophane (sensitivity 1 μ g).

6. Tyrosine Dip Reagent.

Ionograms were dipped in a 0.1% solution of 1-nitroso-2-naphthol (Fisher Certified Reagent) in acetone and were allowed to dry at 25°. Following a second dip treatment with

10% HNO_3 in acetone, the papers were again dried at room temperature. Heating the paper at 90° for 3 minutes revealed tyrosine peptides as red spots on a pale green background (sensitivity 1 μg).

7. Sakaguchi Reaction for Arginine (257).

Arginine peptides were detected by first dipping the ionogram in 0.1% 8-hydroxyquinoline in acetone (Matheson, Coleman and Bell Co.) and drying at room temperature. The paper was lightly sprayed with a solution of bromine/NaOH (0.2 ml of bromine in 100 ml of 0.5 N NaOH) (Mallinckrodt Chemicals). Arginine peptides appears as red spots (sensitivity 0.1 μg).

For more extensive information on the use of detection reagents for other amino acids (e.g. cysteine, cystine, methionine, serine, threonine, glycine, taurine, proline) the reader is referred to Bailey (257) and Block, Durrum and Zweig (260).

Appendix C

Hydrolysis Procedure (233)

1. Proteins:

The protein (~ 1 mg) in a pyrex test tube (18 x 150 mm, 15 x 125 mm) is suspended in 1 ml of 6N HCl (1:1 dilution of reagent concentrated HCl). Using a pair of forceps to grip the rim of the test tube, a section of the tube about 2-3 cm from the top is constricted, in an oxygen flame, to about 1 mm bore. The sample is thoroughly chilled in a bath of solid carbon dioxide and acetone and attached through a short sleeve of Tygon tubing to an oil pump protected with a dry ice/acetone trap. When the tube is evacuated, the solution is allowed to come slowly to room temperature. The tube is shaken periodically to dispel the dissolved air in the solution. When bubbles of air cease to form (after 15 minutes), the tube is sealed under vacuum with the aid of an oxygen flame. The protein is hydrolysed for 20 hours at $110^{\circ} \pm 2^{\circ}$ and stored (without opening) at -20° until required. Opening of the tube is easily accomplished by scratching the surface with a diamond pencil followed by applying a hot glass rod to the scratch. The cut end of the tube is fire-polished and the HCl is removed at 50° in a Buchler rotary Evapo-Mix. In preparation for amino acid analysis, the residue is subjected to air oxidation at pH 6.5 as outlined in Chapter 4. 2a ii.

2. Peptides:

Peptides eluted with water (0.2 ml) from paper ionograms and collected in 10 x 75 mm pyrex test tubes (234) were made to 6N HCl by additional of an equal volume of reagent grade concentrated hydrochloric acid. If the peptides were eluted with solutions other than water, the eluents were first freeze-dried and then hydrolysed in 0.5 ml of 6N HCl. The test tubes were constricted, evacuated, sealed and hydrolysed as outlined earlier. Due to the smallness of the test tubes, the HCl was removed under the vacuum provided by a good oil pump. The residue was dissolved in an appropriate volume of 0.2 N citrate buffer pH 2.2 in preparation for amino acid analyses.

3. Dansyl Tubes:

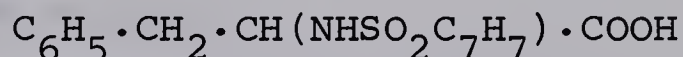
Although Gray and Hartley (206, 212) did not hydrolyse Dansyl-peptides in partially evacuated tubes, such a modification has been employed in the present studies.

Following the addition of $\sim 50 \mu\text{l}$ of 6N HCl to the Dansylated peptide, a thin glass rod was attached to the rim of the small test tube (6 x 30 mm). A section about 0.5 cm from the top of the tube was constricted with the aid of a fine oxygen flame. The glass rod was detached and the tube was briefly immersed in a dry-ice/acetone bath. After the tube was attached to the pump, it was partially evacuated (30 seconds on pump) and sealed with an oxygen flame. Hydrolysis of the Dansyl-peptide was conducted at 110° for 20 hours. The HCl was removed under the vacuum provided by a good oil pump.

Appendix D

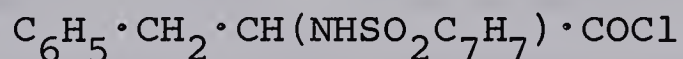
Synthesis of L-1-Tosylamido-2-Phenylethyl Chloromethyl Ketone (L-TPCK)

1. N-tosyl-L-phenylalanine: (194)



L-phenylalanine (10 g., 60.5 mmoles, Sigma Chemicals Lot. 24B-06k0) was dissolved in 130 ml of 1N NaOH. Tosyl chloride (12.1 g., 63.3 mmoles, Eastman Organic Chemicals) in 40 ml of ether was added and the mixture was shaken in a stoppered container for four hours. The emulsion was acidified to congo red and the white precipitate rapidly dissolved into the ethereal layer. The aqueous layer was separated and washed twice with ether. The ether layer and washing were pooled, taken to dryness, and the residue was recrystallized from 60% ethanol. N-tosyl-L-phenylalanine, (15.2 g., m.p. 163 - 164° decomp., literature: M.P. 161°) was obtained in 80% yield. (All melting points are uncorrected).

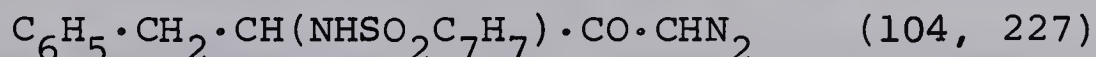
2. N-tosyl-L-phenylalaninyl chloride: (224)



Phosphorous pentachloride (3.9 g., 18.7 mmoles, Baker Analyzed Reagent) was added to N-tosyl-L-phenylalanine (5.4 g., 16.9 mmoles) in 75 ml of anhydrous ether at 0°. The mixture was shaken for 10 minutes at 0°, then for 10 minutes at 25°, and finally stored at 0° for one hour. The crystalline

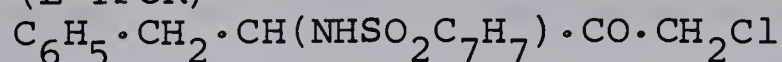
product was filtered off, washed quickly on the Buchner funnel with a little cold ether and then dried for two hours in a vacuum desiccator under the vacuum provide by a good oil pump. N-tosyl-L-phenylalaninyl chloride (4.8 g., m.p. 128 - 129^o decomp. Literature M.P. 128 - 129^o) was obtained in 88% yield.

3. L-1-Tosylamido-2-Phenylethyl Diazomethyl Ketone:



N-Tosyl-L-phenylalaninyl chloride (3.5 g., 11 mmoles) was suspended in 100 ml of ether and chilled in an ice-bath. A cold ethereal solution of diazomethane (19.3 mmoles standardized by titration (196)) was added slowly to the stirred suspension. The slightly yellow solution was allowed to come to room temperature upon standing overnight and was briefly refluxed for 15 minutes. It was important that a molar excess of diazomethane, no greater than 2/1, be present, since at higher concentrations, N-methylation of the acidic hydrogen on the tosylamido group will occur. The order of the addition of the reagent (diazomethane added to the chloride) largely prevented N-methylation and promoted the synthesis of the chloromethyl ketone. The solution containing diazomethane and a mixture of the diazomethyl and chloromethyl ketones was used for the final step of the synthesis.

4. L-1-Tosylamido-2-Phenylethyl Chloromethyl Ketone (L-TPCK)



Anhydrous hydrogen chloride gas (Matheson of Canada Ltd., Whitby, Ontario) was bubbled through the ethereal

solution present after step 3 for one hour. The ether was evaporated and recrystallization of the residue from ethanol (95%) gave colorless crystals of L-TPCK (2.4 g., m.p. 99-101) in 69% yield. (Literature: 102 - 103^o (104)).

Analyses calculated for C₁₇H₁₈ClNO₃S: C, 58.03; H, 5.16; Cl, 10.08; N, 3.98; O, 13.64; S, 9.12. *Found: C, 60.68; H, 5.55; Cl, 4.63; N, 3.98; O, 15.72; S, 9.46. The observed discrepancies in the elemental analyses were traced to the presence of a hydroxymethyl ketone by-product (C₆H₅·CH₂·CH(NHSO₂C₇H₇)·CO·CH₂OH) in the L-TPCK preparation. The synthesis presented is the corrected version and omits a detrimental sodium bicarbonate (10%) washing of the HCl-ethereal solution of L-TPCK. This washing undoubtedly led to the undesirable side reactions. In all subsequent syntheses of other chloromethyl ketones, bicarbonate washings were avoided.

Synthesis of D-l-Tosylamido-2-Phenylethyl Chloromethyl Ketone (D-TPCK)

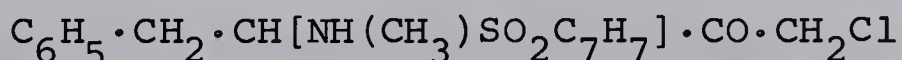
The synthesis of D-TPCK was performed in a manner analogous to that for the L-isomer as reported by Schoellman et al. (104). N-tosyl-D-phenylalanine, formed from D-phenylalanine (Sigma Chemical Lot P38-53) and tosyl chloride had a melting point of 164 - 165^o. A mixed melting point of D- and L-N-tosyl-phenylalanine was found to be 139 - 141^o. (Literature: 134 - 135^o). N-tosyl-D-phenylalaninyl chloride, synthesized according to Popenoe and du Vigneaud (224) was found to have a melting point of 129 - 130^o (decomp.)

*Elemental analyses were performed by Dr. C. Daessle, Organic Microanalyses, 5757 Decelles Ave., Montreal, Quebec.

D-TPCK was crystalized from ethanol (95%) and gave colorless crystals with a melting point of 104-105° in 65% yield.

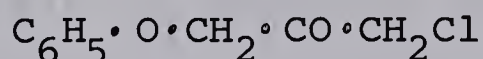
Analyses calculated for $C_{17}H_{18}ClNO_3S$: C, 58.03; H, 5.16; Cl, 10.08; N, 3.98; O, 13.64; S, 9.12. Found: C, 58.28; H, 5.16; Cl, 10.35; N, 3.77; O, 13.41; S, 9.30.

Synthesis of L-1-N-Tosyl-N-Methylamido-2-Phenylethyl Chloromethyl Ketone (N-Methyl-L-TPCK)



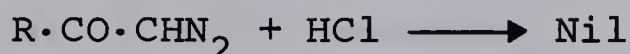
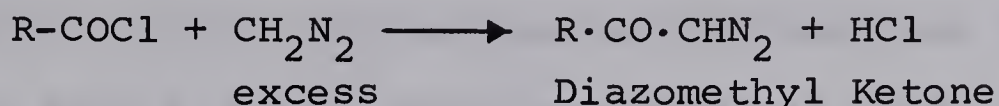
The synthesis of N-methyl-L-TPCK was performed according to the method of Schoellman et al. (104). A molar ratio of $CH_2N_2/R-COCl$ of 9 to 1 was employed in order to cause N-methylation of the acidic hydrogen of the tosylamido group in addition to the formation of the diazomethyl ketone (226). Recrystallization of the product from ethanol (95%) gave crystals of melting point 105 - 107°, in 70% yield (Literature: 104 - 106° (104)). Analyses calculated for $C_{18}H_{20}ClNO_3S$: C, 59.09; H, 5.51; Cl, 9.69; N, 3.83; Found: C, 60.12; H, 4.94; Cl, 9.82; N, 3.46.

Synthesis of Phenoxyethyl Chloromethyl Ketone (PMCK)



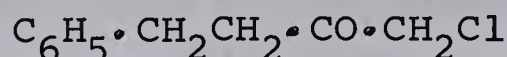
Phenoxyacetyl chloride (6.8 g., 39 mmoles, Eastman Organic Chemicals) was placed in a liter flask and immersed in an ice-bath. A cold ethereal solution of diazomethane (50 mmoles) was slowly added (10 minutes) in order to control the evolution of nitrogen gas. The stoppered flask was allowed to stand overnight in the fume hood. If the phenoxy-methyl diazomethyl ketone is desired, then the phenoxyacetyl

chloride should be added in a drop-wise manner to an excess of diazomethane.



Anhydrous hydrogen chloride gas was bubbled through the solution until the yellow-color had disappeared. The disappearance of color indicated the destruction of the diazo groups by HCl gas. After removing the solvent, the residue was dissolved in 400 mls of hot petroleum ether (Fisher Certified Reagent, boiling range 35.5° to 53.7°). Rapidly cooling the solution in a dry-ice - acetone bath accompanied by vigorous scratching of the container walls, caused the precipitation of crystals which were collected on a Buchner funnel. Drying the product under vacuum gave 4.5 g of colorless crystals (m.p. 33 - 34°) in 66% yield. PMCK was stored at -20°C until required. Analyses calculated for C₉H₉ClO₂: C, 58.55; H, 4.91; Cl, 19.21; O, 17.33. Found: C, 58.29; H, 4.83; Cl, 19.49; O, 17.51.

Synthesis of β-Phenylethyl Chloromethyl Ketone (βPECK)



An ethereal solution of diazomethane (70 mmoles) was slowly added to hydrocinnamoyl chloride (6.74 g., 40 mmoles, Eastman Organic Chemicals) in a liter flask chilled in an ice-bath. Following the evolution of nitrogen gas the solution was stoppered and allowed to come to room temperature

overnight. Anhydrous hydrogen chloride gas was bubbled through the solution until it became colorless (20 minutes). Evaporation of the solvent and crystallization from 60% ethanol gave 4.2 g of colorless crystals (m.p. 40 - 41°; Literature: 39 - 40° (225)) in 57% yield. β PECK was stored at 3° until required.

Analyses calculated for $C_{10}H_{11}ClO$: C, 65.76; H, 6.07; Cl, 19.41; O, 8.76. Found: C, 65.90; H, 5.96; Cl, 19.14; O, 9.06.

Synthesis of Benzyl Chloromethyl Ketone (BCK)

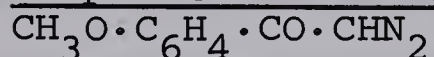


To phenylacetyl chloride (4.65 g. 30 mmoles, Eastman Organic Chemicals) in a liter flask at 0° was slowly added a cold ethereal solution of diazomethane (60 mmoles). Following the rapid evolution of nitrogen, the flask was stoppered and the reaction was allowed to proceed overnight at room temperature. Anhydrous hydrogen chloride gas was bubbled through the yellow solution until the color disappeared (20 minutes). Subsequent to removal of the solvent, the residue was crystalized from 400 ml of hot petroleum ether by cooling in a dry-ice acetone bath accompanied by vigorous scratching of the container walls. Benzyl chloromethyl ketone was collected on a cold Buchner funnel (-10°) and stored at -20° until required. The product (3.1 g., m.p. 15 - 16°) was obtained in 61% yield. Analyses calculated for C_9H_9ClO : C, 64.11; H, 5.38; Cl, 21.03; Found: C, 64.28; H, 5.32; Cl, 21.62.

Synthesis of Anisoyl Chloromethyl Ketone (ACK)



1. Preparation of anisoyl diazomethyl ketone

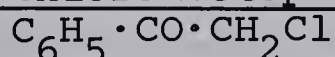


Anisoyl chloride (6.85 g., 40 mmoles, Eastman Organic Chemicals) was added dropwise to a cold ethereal solution of diazomethane (137 mmoles) and the reaction was allowed to go to completion upon standing overnight at room temperature. Removal of the ether by distillation and recrystallizing the residue from benzene-petroleum ether led to the recovery of anisoyl diazomethyl ketone as yellow crystals (4.45 g, m.p. 89-90° decomp. Literature: 90 - 91° (197)) in 65% yield.

2. Preparation of anisoyl chloromethyl ketone.

Dry hydrogen chloride gas was bubbled through an ethereal solution of anisoyl diazomethyl ketone (1.5 g) for 30 minutes. The residue remaining after the solvent was removed was recrystallized from ethanol (95%) and gave colorless crystals (0.93 g., m.p. 98 - 100°) in 62% yield. Analyses calculated for $\text{C}_9\text{H}_9\text{ClO}_2$: C, 58.55; H, 4.91; Cl, 19.21. Found: C, 58.38; H, 4.72; Cl. 19.48.

α -Chloroacetophenone (CA)



This reagent was purchased from Eastman Organic Chemicals and was used without further purification.

Preparation of Alcohol-Free Ethereal Solutions of Diazomethane (CH_2N_2) (191-193, 226, 227)

"Carbitol" (diethyleneglycol monethyl ether, Union Carbide Chemical Co.) or 2-(2-ethoxyethoxy)-ethanol (Eastman Organic Chemicals) (35 ml) and ether (10 ml) were added to

a solution of potassium hydroxide (6 g.) in water (100 ml). The alkali solution was contained in a 300 ml three-necked, round-bottom flask fitted with a dropping-funnel and an efficient condenser set downward for distillation. The condenser was connected to two receiving flasks in series containing 20 and 35 ml of ether, respectively.

Potassium hydroxide pellets were introduced into both flasks to act as drying agents. The inlet tube of the receivers dipped below the surface of the ether and the flasks were cooled in an ice-bath. The distilling flask was heated in a water-bath at 70°. As the distillation of the ether commenced, a solution of 21.5 g. of "Diazald" (N-methyl-N-nitroso-p-toluenesulfonamide, (191) Aldrich Chemical Company) in 150 ml of ether was added through the dropping funnel over a 20 minute period.

The rate of distillation was about equal to the rate of addition, the flask being shaken occasionally. When the dropping funnel was empty, another 20 ml of ether was slowly added and the distillation was continued until the distilling ether was colorless. The pooled yellow ethereal solution from the receiving flask contained about 3 g. (\sim 70 mmoles) of diazomethane. Prior to use, the cold diazomethane solution was dried over potassium hydroxide pellets for at least two hours and was titrated in the manner described below.

It should be noted that diazomethane is a highly toxic yellow gas and certain precautions should be taken during its preparation and use. All manipulations should be conducted

in a fume hood equipped with an efficient exhaust system. Cooled dilute ethereal solutions are "relatively" safe to handle. The use of ground glass apparatus is not recommended, but if it is employed, care should be taken that all surfaces are coated with vacuum grease (Dow-Corning high vacuum). The use of a glass stirrer within a glass sleeve, where the grinding action could produce glass dust and a rough surface, is to be strictly avoided. In an emergency, decomposition of diazomethane may be rapidly effected with hydrochloric acid.

Titration of Ethereal Solutions of Diazomethane
(196)

An aliquot (5 ml) of the cold, dry diazomethane solution was allowed to react with an accurately weighed amount of benzoic acid (300 mg) in ether (50 ml). The transformation of a portion of the benzoic acid to methyl benzoate occurred rapidly as witnessed by the disappearance of the yellow color and the evolution of nitrogen gas. The solution was diluted with about 10 ml of water and the benzoic acid remaining was titrated with 0.2 M NaOH using phenolphthalein as an indicator. Subtracting the mmoles of benzoic acid titrated from the amount of benzoic acid originally present yielded the number of mmoles of diazomethane in the aliquot. Suitable volumes of the ethereal diazomethane were then used to give the desired molar ratio with the various acyl chlorides employed in the syntheses.

Synthesis of S-(β -Aminoethyl)-L-Cysteine

The synthesis of S-(β -aminoethyl)-L-cysteine

($\text{H}_2\text{N}\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{S}\cdot\text{CH}_2\cdot\text{CH}(\text{NH}_2)\text{COOH}$) was performed according to the method of Cavallini et al. (200), but with the following modifications: L-cysteine hydrochloride (5 g) was dissolved in 10 ml of water through which nitrogen had been bubbled for 15 minutes. An aqueous solution of KOH (35%), bubbled with nitrogen, was added followed by the addition of 1.8 ml (1.5 g) of ethylenimine (molar ratio ethylenimine/-SH equals 1.1/1). The reaction was allowed to proceed for 15 minutes under N_2 at 25° and the solution was neutralized with concentrated HCl. Ethanol was not required since the use of ethylenimine did not introduce bromide ions as did bromoethylamine • HBr utilized by Cavallini et al. (200). Subsequent purification and crystallization were adopted as presented by these workers (200).

The product (3.7 g) was isolated in 50% yield and possessed a melting point of $195 - 196^\circ$ (Literature $192 - 192.5$ (200)).

Calculated: C, 29.93; H, 6.47; N, 13.96; S, 15.97

Found: C, 30.24; H, 6.93; N, 14.09; S, 16.58

In preparation for the determination of the integration constant (C), a portion of S-(β -aminoethyl)-L-cysteine was dried to constant weight at 100° under the vacuum provided by a good oil pump. Aliquots of an accurately prepared stock solution were analysed on the 20 cm basic column of the Beckman 120 B amino acid analyzer equipped with the accelerated system of Spackman et al. (239). Under these conditions AE-cysteine was eluted in the 120 minute

position whereas lysine and histidine were eluted in the 110 minute and 133 minute position, respectively. Through use of the equation -

$$\mu\text{moles} = \frac{\text{height} \times \text{width of the peak}}{\text{integration constant (C)}}$$

an integration constant of 46.6 was determined from the average of five analyses.

Hydrolysing aliquots of a stock solution of AE-cysteine (110° , 6N HCl) in a sealed evacuated tube at various intervals over 72 hours and analysing the hydrolysate as before, lead to complete recovery of AE-cysteine. The stability of AE-cysteine to HCl at 110° has also been demonstrated in the presence of a protein hydrolysate.

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